REMARKS

Claims 16, 18-20, 32-39 and 157-165 were pending in the subject application. Applicants have amended claims 16, 20, 32 and 33 and canceled claim 18. Claims 157-165 have been withdrawn by the Examiner. Accordingly, claims 16, 19-20 and 32-39 are currently pending.

In section 1 of the January 29, 2004 final Office Action, the Examiner indicated that all previous grounds of rejection have been withdrawn in view of Applicant's amendment filed October 17, 2003. The Examiner, however, has applied new grounds of rejection not necessitated by Applicant's amendment and made the present Office Action NON-FINAL.

In section 2 of the January 29, 2004 final Office Action, the Examiner rejected claims 16, 18-20 and 32-39 under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Examiner alleged that the claims are drawn to a "pharmaceutical composition" for the treatment of "an autoimmune disease." The Examiner further alleged that Claim 39 provides an extensive list of autoimmune diseases characterized by different disease etiologies and reactivities to various autoantigens. The Examiner asserted that the meaning of the term "pharmaceutical composition" is understood to imply a composition whose sole purpose is for administration to a subject for the treatment of a condition and as a composition with a specified use and that claims to a "pharmaceutical composition" must meet a level of enablement commensurate with that needed for a therapeutic method. The Examiner stated the effectiveness of treating a response to an autoantigen is dependent on several factors, the most critical of which is whether the therapy can be used to treat an

ongoing autoimmune response or whether it is only effective prophylactically, citing to Tisch, et al., Proc. Nat. Acad. Sci. (USA), 1994, 91:437-438, ("Tisch"). The Examiner alleged that typically, an autoimmune disease is diagnosed only after significant tissue damage has already occurred and that administration of an antigen after pathogenic T cells have been activated may have an exacerbating effect on the disease, rather than a tolerogenic one. The Examiner also alleged that another problem during the treatment of autoimmune diseases is determinant spreading during the course of the disease, pointing out that Tisch also teaches that "the high degree of specificity required for the process of clonal deletion/anergy may be limiting when dealing with diseases such as MS, IDDM, and RA, in which there are responses to several autoantigens [...] and the critical inciting autoantigen(s) is not known" (page 437, third full paragraph of column 3 in particular). The Examiner alleged that the breadth of applicant's claim is such that it recites a composition for the treatment of unrelated autoimmune diseases with a random-sequenced peptide terpolymer of a similar amino acid composition to myelin basic protein (MBP), an antigen related to the etiology of multiple sclerosis (MS) and the animal model experimental allergic encephalomyelitis (EAE). The Examiner stated that the specification demonstrates that prophylactic incubation of cells with the terpolymer inhibits T cell proliferation in response to MBP (Example 6) and inhibits a collagen-specific T cell response (Example 9). The Examiner concluded that specification does not, however, indicate that any of these diseases, including MS, could be successfully treated with the terpolymer of the invention, as in each case the examples show only prophylactic success in inhibiting a response of a previously characterized T cell line to a single well-defined antigen and does not address the effect of an ongoing

autoimmune condition where reactivity is directed to multiple antigenic epitopes. The Examiner referred to Example 10 of the instant specification and alleged that copolymer 1 inhibits activation of T cells reactive with a single antigenic epitope of the acetylcholine receptor (AChR). However, the Examiner noted, myasthenia gravis (MG) is well known by practitioners to involve reactivity to a plurality of antigenic epitopes on the AChR, not a single epitope, and that the epitopes recognized can vary greatly between MG patients. Therefore, the Examiner asserted that, based upon the lack of guidance in the instant specification, an artisan would not be able to predict any specific autoimmune diseases that would be treatable with a pharmaceutical composition of the present invention.

The Examiner concluded that in view of the nature of the invention, the state of the art, the limited working examples, the unpredictability of the art, the lack of sufficient guidance in the specification and the breadth of the claims, it would take undue trials and errors to practice the claimed invention and this is not sanctioned by the statute.

In response, applicants respectfully traverse the Examiner's rejection on the basis that the examples provided in the applicants' specification enable the applicants' amended claims.

As an initial matter, applicants note that the Examiner has cited no authority for imposing "the level of enablement commensurate with that needed for a therapeutic method" on "pharmaceutical composition" claims. Applicants respectfully request that the Examiner either cite to a basis supporting this analysis, or withdraw the rejection using the analysis.

Furthermore, because the Examiner has analyzed the claims as if they were method claims, applicants do not understand the Examiner's rational for the withdrawal of actual method claims 157-165. Applicants contend that if the Examiner continues to rely on a standard applicable to method claims, withdrawal of method claims is improper.

Applicants now address the Examiner's rejection, as stated in the January 29, 2004 Office Action by respectfully directing the Examiner's attention to MPEP § 2164.02:

> The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. **An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention**. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, **if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as such unless the examiner has evidence that it does not correlate. Even with such evidence, the examiner must weigh the evidence** for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995)(reversing the PTO decision based on

finding that *in vitro* data did not support *in vivo* applications)(emphasis added).

The applicants have provided in the specification several recognized models (e.g., EAE suppression, inhibition of the response of collagen specific T cells and Myasthenia Gravis Antigenic peptide T cells) that correlate the performance of the pharmaceutical compositions to the treatment of diseases recited in the claims (e.g., multiple sclerosis, rheumatoid arthritis and Myasthenia Gravis). These specific diseases are representative of autoimmune diseases generally. In addition, applicants have also provided in the specification HLA binding results for the claimed composition which is a working model that correlates to autoimmune diseases generally.

The correlation between the EAE model and multiple sclerosis is well established in the art as is evidenced, e.g., by Arnon, et al., Israel J. Med. Sci., 1989, 25:686-689, ("Arnon"). For example, Arnon establishes a correlation between suppression of EAE in an *in-vivo* animal model by copolymer 1 and treatment of patients afflicted with multiple sclerosis by copolymer 1. Arnon also shows the stimulation of MBP specific T cells as well as the inhibition of MBP-induced T cell proliferation. Finally, Arnon describes the cross reactivity between anti-MBP antibodies and copolymer 1. By contrast, the Examiner has presented no evidence that the EAE model and multiple sclerosis do not correlate.

Applicants have also shown that mixtures of terpolymers suppress EAE (Example 3, pages 30-34 of the subject application), inhibit MBP-induced T cell proliferation (Example 6, pages 43-46 of the subject application), stimulate COP-1 specific T cells (Example 7, pages 46-48 of the subject

application), and cross react with anti-COP-1 antibodies (Example 8, pages 48-51 of the subject application). Based on these working models and the correlation between the working models and the treatment of multiple sclerosis with copolymer 1, one skilled in the art would be able to make a pharmaceutical composition comprised of the mixtures of terpolymers for the treatment of multiple sclerosis without undue experimentation. Therefore, the subject application does enable one skilled in the art to make a pharmaceutical composition for the treatment of multiple sclerosis.

Rheumatoid arthritis has been strongly associated with human leukocyte antigens HLA-DR1 and HLA-DR4, (Fugger, et al. Arthritis Research 2000, 2: 208-211, a copy of which is enclosed herewith as **Exhibit 1**). Moreover, type II collagen is known to induce arthritis in animal models and molecules that compete with collagen in binding to HLA-DR molecules and which inhibit collegan specific T cells response have been previously proposed as compounds for treating rheumatoid arthritis (Fridkis-Hareli, PNAS, 1998, Vol. 95, pp 12528-31, a copy of which is enclosed herewith as **Exhibit 2**).

Applicants have shown that the terpolymers of the subject application bind to HLA-DR1 and HLA-DR4 (Example 5, pages 37-43 of the subject application). Applicants have also shown that the terpolymers compete with collagen for binding to human leukocyte antigens and inhibit collagen-specific t-cell response (Page 17, line 34 to page 18, line 1 and Example 9, page 52 of the subject application). Based on these working models and the correlation between the working models and compounds previously proposed for treating rheumatoid arthritis, one skilled in the art would be able to make a pharmaceutical composition comprised of the terpolymers for

the treatment of rheumatoid arthritis without undue experimentation. Therefore, the subject application does enable one skilled in the art to make a pharmaceutical composition for the treatment of rheumatoid arthritis.

Human leukocyte antigens and myasthenia gravis have also been correlated, (Giraud, et al., Neurology, 2001 57(9): 1555-60). The acetylcholein receptor as a model for myasthenia gravis is known in the art (Boyton, et al., Clin. Exp. Immunol., 2002, 127: 4-11). Applicants have shown that copolymer 1 inhibit T Cells responsive to a Myasthenia Gravis Antigenic peptide (Example 10, pages 56-58 of the subject application). As mentioned above, terpolymers also bind to HLAs. Therefore, the subject application does enable one skilled in the art to make a pharmaceutical composition for the treatment of Myasthenia Gravis.

In addition to multiple sclerosis, rheumatoid arthritis and Myasthenia Gravis, applicants have provided a reasonable expectation that their claimed pharmaceutical composition would treat other autoimmune diseases. Human leukocyte antigens (HLA) are part of the Major Histocompatibility Complex (MHC) and therefore key elements of the immune response, (Mallios, Bioinformatics, 1999, Vol. 15, No. 6, 432-439, a copy of which is enclosed herewith as **Exhibit 3**). The associations of HLA and specific autoimmune diseases are known and presented for the Examiner's convenience in tabular form in **Exhibit 4**. For example, as stated above, rheumatoid arthritis is associated with HLA-DR 1 and 4. Autoimmune thyroiditis has a known association with HLA-DR3, (Wan, et al., Hum. Immunol. 2002, Apr, 63(4):301-10, a copy of the abstract of which is enclosed herewith as **Exhibit 5**). A number of autoimmune diseases are associated with HLA-DR4: autoimmune

uveoretinitis, (Giuseppina, et al., The Journal of Clinical Investigation, April 2003, Vol. 111, No. 8, 1171-80, a copy of which is enclosed herewith as **Exhibit 6**), colitis, (Kobayashi, et al., Clin Exp Immunol. 1990 Jun; 80(3):400-3, a copy of the abstract of which is enclosed herewith as **Exhibit 7**), diabetes mellitus, (http://ntri.tamuk.edu/immunology/autoimmunity.html, a copy of which is enclosed herewith as **Exhibit 8**), Graves disease, (Sridama, et al., *Arch Intern Med.* 1987;147:229-231, a copy of the abstract of which is enclosed herewith as **Exhibit 9**), Hashimoto's disease, (Lymberi, et al., Arch Hellen Med, 16(4), July-August 1999, 337-351, a copy of the abstract of which is enclosed herewith as **Exhibit 10**), psoriasis, (Fatma, et al., Swiss Med Wkly, 2003, 133: 541-543, a copy of which is enclosed herewith as **Exhibit 11**), pemphigus vulgaris, (Lombardi, et al., J Invest Dermatol. 1999 Jul;113(1):107-10, a copy of the abstract of which is enclosed herewith as **Exhibit 12**), and systemic lupus erythematosus, (Batchelor, et al. Lancet 1980 1(8178):1107-9, a copy of the abstract of which is enclosed herewith as **Exhibit 13**). As discussed above with rheumatoid arthritis, binding to HLA-DRs has been correlated with molecules that treat autoimmune diseases.

Applicants have shown that their mixtures of terpolymers bind to HLA-DR 1, 2, and 4, (Example 5, pages 37-43 of the subject application). Since applicants have provided working examples that correlate with several autoimmune diseases as well as the binding data examples to HLA-DR 1, 2 and 4 that correlate with numerous other autoimmune diseases, the applicants maintain that they have provided a reasonable expectation that their mixtures of terpolymers treat autoimmune diseases. Importantly, the Examiner offers no evidence that applicants' models do not correlate to the claims.

Yet, furthermore, applicants point out that Tisch also refered to by the Examiner, describes EAE as a "model of autoimmunity" (page 438, column 1, paragraph 2). Suppressor cell activity has been shown in the EAE model in mice and rats and has been suggested in other autoimmune diseases (Aharoni et al., Eur. J. Immunol., 1993, 23, 17-25, a copy of which is enclosed herewith as **Exhibit 14**). Relapses and remissions often occur in multiple sclerosis and in other autoimmune diseases and these relapses and remissions have been explained by changes in the suppressor cell population (*Id* at p17). Thus, EAE and autoimmune diseases involve at least some of the same mechanisms. Therefore, EAE is an appropriate model for autoimmune diseases. Applicants, however, provide more and other models as well.

Applicants further point out, as the Examiner is aware, that the enablement of a representative number of species within a genus enables the genus (MPEP § 2164.02). Applicants have certainly enabled treatment of a representative number of autoimmune diseases. Accordingly, applicants respectfully submit that the enablement requirement is satisfied for the claims as amended.

In light of the above comments, applicants request that the Examiner reconsider and withdraw the rejection of claims 16, 18-20 and 32-39 under 35 U.S.C. 112, first paragraph.

In section 3 of the January 29, 2004 final Office Action, the Examiner rejected claim 18 under 35 U.S.C. 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner alleged that the term "substantially free" in claim 18 is a relative term that renders the claim indefinite. The Examiner further alleged that the term "substantially free" is not defined by the claim, the specification does not appear to provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. The Examiner questioned whether the term refers to a function of the cited constituent or does it refer to the physical presence thereof? The Examiner further questioned if "substantially free" is referring to a physical presence, what ratio or amount of the constituent constitutes an upper limit for "substantially free"?

In response, without conceding the correctness of the Examiner's comments and solely to advance the prosecution of the subject application, the applicants have canceled claim 18 without disclaimer or prejudice.

In section 4 of the January 29, 2004 final Office Action, the Examiner rejected claims 16, 18-20 and 32-39 under 35 U.S.C. 102(b) as allegedly anticipated by Arnon, et al., Israel J. Med. Sci., 1989, 25:686-689, ("Arnon").

The Examiner alleged that Arnon teaches COP 1, a synthetic basic random copolymer comprising A, E, K, and Y residues. The Examiner reminded the applicant that the term "comprising" in claim 16 is an open term that allows the inclusion of other elements that are not specifically recited in the claim, including glutamic acid residues. The Examiner added that the phrase "consisting essentially of" in claim 16 is being interpreted as inclusive or open-ended, not excluding additional non-recited elements, i.e., "comprising," provided

that the additional elements do not materially affect the basic and novel characteristic(s) of the claimed invention. The Examiner included claim 18 because allegedly the term "substantially free" is a relative term that has not been adequately defined by the instant specification or claims as filed. The Examiner stated that Arnon teaches that alanine is present in the polymer at a molar ratio of 6.0, glutamic acid is present in the polymer at a molar ratio of 1.9, lysine is present in the polymer at a molar ratio of 4.7 and tyrosine is present in the polymer at a molar ratio of 1.0 (Abstract in particular) and concluded that given the sum of the molar ratios is 13.6, alanine is present as a molar fraction of 0.441, lysine is present as a molar fraction of 0.346 and tyrosine is present as a molar fraction of 0.140 [claim 19]. The Examiner also included claim 20 because the term "about" is a relative term and the metes and bounds of the term have not been established by the specification. According to the Examiner, 0.441 satisfies the recitation of "about 0.54," 0.346 satisfies the recitation of "about 0.35" and 0.140 satisfies the recitation of "about 0.10." The Examiner alleged that Arnon teaches that COP 1 was effective in exerting a suppressive effect on EAE when injected into guinea pigs when administered in an aqueous saline solution, a pharmaceutically acceptable carrier (page 686, second column in particular) and further states that COP 1 is effective in suppressing EAE in rabbits, mice, rhesus monkeys and baboons (page 686, second column in particular). The Examiner concluded that prior art teaching anticipates the claimed invention.

The Examiner stated that claims 32 and 33 are included because, while Arnon allegedly does not specifically teach the size of COP 1, it is noted that it is a randomly arranged synthetic polypeptide product and the final size of the product

will be determined by the amounts of the individual constituent amino acid residues added to the reaction mixture and the time the reaction is allowed to run. Furthermore, the Examiner alleged that in order to exert its effect on T cells, the peptide must be processed into small 8-20 amino acid residue long epitope peptide, irrespective of the starting size of the polypeptide. According to the Examiner, provided that the ratio of the elements is maintained in the synthesis of the polypeptide, the beginning size of the polymer is not seen as being patentably distinct.

In response, applicants direct the Examiner's attention to page 1, lines 27-34 of the subject application where terpolymers are defined, "When such mixtures of synthetic random linear copolymers consist essentially of the three of the four amino acids found in Copolymer 1, they are referred to as Terpolymers...Preferably, the Terpolymers are composed of tyrosine, alanine and lysine,...". Applicants also point out that claim 16 recites "consisting essentially of randomly polymerized tyrosine, alanine and lysine..." However, Arnon does not disclose tyrosine, alanine and lysine polymers but rather discloses glutamic acid, tyrosine, alanine and lysine polymers.

By alleging that Arnon anticipates the claims of the subject application, the Examiner has impermissibly broadened the scope of claim 16. *SciMed Life Systems, Inc. V. Advanced Cardiovascular Systems, Inc.*, 242 F.3d 1137, 1141 (Fed. Cir. 2001)

> Where the specification makes clear that the invention does not include a particular feature, that feature is deemed to be outside the reach of the claims of the patent, even though the

> language of the claims, read without reference to the specification, might be considered broad enough to encompass the feature in question.

Without conceding the correctness of the Examiner's allegations that the molar fractions of tyrosine, alanine and lysine disclosed in Arnon satisfy the recitation of the molar fractions for these amino acids in claim 20 and solely to advance the prosecution of the subject application, applicants have amended claim 20 to remove the term "about".

While applicants do not understand the Examiner's point regarding claims 32-33 and the length of the polypeptides, applicants maintain that the distinction between Arnon and the invention of the subject application is not the length of the terpolymers but rather their composition.

Accordingly, Arnon does not anticipate the subject invention and the Examiner is respectfully requested to withdraw his rejections of claims 16, 19-20 and 32-39 under 35 U.S.C. 102(b) as being anticipated by Arnon.

In section 5 of the January 29, 2004 final Office Action, the Examiner rejected claims 16, 18-20, and 32-39 under 35 U.S.C. 103(a) as allegedly unpatentable over Teitelbaum, et al. (Proc. Nat..Acad. Sci. [1988] 85(24):9724-9728 ("Teitelbaum") in view of Arnon.

The Examiner alleged that Teitelbaum teaches Cop 1, a 21,000 dalton synthetic basic random copolymer comprising A, E, K, and Y residues. The Examiner reminded the applicants that the term "comprising" in claim 16 is an open term that allows the

inclusion of other elements that are not specifically recited in the claim, including glutamic acid residues. The Examiner added that the phrase "consisting essentially of" in claim 16 is being interpreted as being inclusive or open-ended, not excluding additional non-recited elements, i.e., "comprising," provided that the additional elements do not materially affect the basic and novel characteristic(s) of the claimed invention. The Examiner included claim 18 because the term "substantially free" is a allegedly a relative term that has not been adequately defined by the instant specification or claims as filed. The Examiner alleged that Teitelbaum teaches that alanine is present in the polymer at a molar ratio of 6 0, glutamic acid is present in the polymer at a molar ratio of 1.9, lysine is present in the polymer at a molar ratio of 4.7 and tyrosine is present in the polymer at a molar ratio of 1.0 (Abstract in particular) and concluded that given the sum of the molar ratios is 13.6, alanine is present as a molar fraction of 0.441, lysine is present as a molar fraction of 0.346 and tyrosine is present as a molar fraction of 0.140 [claim 19].

The Examiner stated that claim 20 is included because the term "about" is a relative term and the metes and bounds of the term have not been established by the specification. The Examiner alleged that 0.441 qualifies as "about 0.54," 0.346 qualifies as "about 0.35" and 0.140 qualifies as "about 0.10." The Examiner alleged that Teitelbaum teaches that Cop 1 was effective in specifically inhibiting T cell responses to myelin basic protein, which is a target autoantigen in the inflammatory autoimmune disease multiple sclerosis and in the experimental allergic encephalomyelitis (EAE) model (see entire publication).

The Examiner acknowledged that Teitelbaum does not teach an "effective amount" in a pharmaceutically acceptable carrier. However, the Examiner alleged that Arnon teaches that the administration of Cop 1 to rhesus monkeys and baboons even after the onset of clinical symptoms of EAE demonstrated reversal of disease symptoms and full recovery (paragraph bridging pages 686-687 in particular) and that the use of Cop 1 in human subjects improved the disability status of the subjects and reduced exacerbation versus placebo-treated controls (paragraph bridging pages 688-689 in particular). The Examiner alleged that Arnon teaches the use of an effective amount of Cop 1.

The Examiner concluded it would have been *prima facie* obvious to a person having ordinary skill in the art at the time the invention was made to formulate an effective amount of the Cop 1 copolymer of Teitelbaum in a pharmaceutically acceptable carrier with a reasonable expectation of success because Arnon teaches that it improves the clinical status of non-human EAE subjects and human multiple sclerosis patients The Examiner alleged that one would have been motivated to use this compound in a pharmaceutical preparation by the teaching of Arnon that Cop 1 is related to the encephalogenic myelin basic protein but is not itself encephalogenic.

The Examiner stated that claim 33 is included because allegedly, while Teitelbaum teaches that Cop 1 is 21 Kd in size, the Examiner alleged that it is a randomly arranged synthetic polypeptide product. The Examiner further alleged that in order to exert its effect on T cells, the peptide must be processed into small 8-20 amino acid residue long epitope peptide, irrespective of the stated size of the polypeptide. The Examiner also

alleged that the ratio of the elements is maintained in the synthesis of the polypeptide, and the beginning size of the polymer is not patentably distinct.

In response, applicants again point out that claim 16 as amended recites "consisting essentially of randomly polymerized tyrosine, alanine and lysine..." Teitelbaum and Arnon, together or individually, do not suggest or motivate the development of a pharmaceutical composition for the treatment of autoimmune diseases which comprises a mixture of terpolymers consisting essentially of tyrosine, alanine and lysine. Finally, neither Teitelbaum nor Arnon teach or suggest a treatment with tyrosine, alanine and lysine terpolymers. Accordingly, the subject invention is patentably distinct and the Examiner is respectfully requested to withdraw his rejections of claims 16, 19-20 and 32-39 under 35 U.S.C. 103(a) as being unpatentable under over Teitelbaum in view of Arnon.

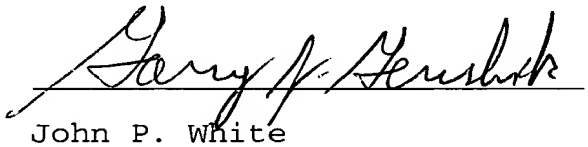
In Section 5 of the January 29, 2004 final Office Action, the Examiner objected to claim 20 as allegedly dependent upon a rejected base claim. However the Examiner stated that the claim would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

In response, applicants point out that claim 20 now depends on a patentable claim 16 as amended.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the enclosed $110.00 for the one-month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is deemed necessary, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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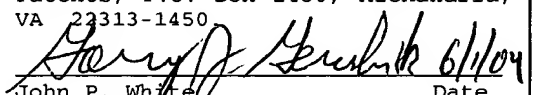

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Review

Association of MHC and rheumatoid arthritis
HLA-DR4 and rheumatoid arthritis: studies in mice and men

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Abstract

Inherited susceptibility to rheumatoid arthritis (RA) is asociated with the DRB1 genes encoding the human leukocyte antigen (HLA)-DR4 and HLA-DR1 molecules. Transgenic mice expressing these major histocompatibility complex (MHC) class II molecules have been developed to generate humanized models for RA. The relevance of these models for understanding RA will be discussed.

Keywords: human leukocyte antigen, rheumatoid arthritis, transgenic mice

Introduction

More than 20 years ago, Stastny [1] reported that HLA-DR4 is associated with RA. Nine years later, Gregersen *et al* [2] proposed the shared epitope (SE) hypothesis based on the observation that the RA-associated DRB1 alleles encode a common sequence of amino acids corresponding to residues 67–74. Several SE-positive (SE+) DRB1 alleles have since been reported to be associated with RA and include the DR4 subtypes DRB1*0401, *0404, *0405 and *0408 as well as the DRB1*0101, *1402, and *1001 alleles. Nepom [3] has summarized the relative risk estimates for Caucasians for three of the most frequent SE+ DRB1 alleles in the Caucasian population. The relative risk is 6 for the DRB1*0401 allele, 5 for the DRB1*0404 allele and 1 for the DRB1*0101 allele. Thus, while the relative risk for individuals carrying the DRB1*0401 allele or the *0404 allele is approximately five times higher compared with that for individuals not carrying these alleles, the DRB1*0101 allele does not confer risk on its own. It has been debated whether the RA-associated DRB1 alleles are disease risk genes or prognostic markers for a more progressive disease course, and whether individuals carrying two SE+ DRB1 alleles either have a higher risk of developing disease or develop more severe disease compared with individuals with only one SE+ DRB1 allele (for recent reviews, see for example [3,4]). So far, no consensus has been reached in answering these questions, which have been addressed most recently in an unselected population based study of 680 new-onset cases with inflammatory polyarthritis, of whom 404 fulfilled the American College of Rheumatology (ARC) criteria for RA [5]. Such a study seems optimal to answer these questions. The study confirmed the association between RA and the presence of any SE allele, and thus demonstrated that the SE allele does in fact confer susceptibility to RA even though the relative risk was modest (RR = 2.3). Both of the two most frequent DRB1*04 alleles, *0401 and

CIA = collagen-induced arthritis; CII = collagen type II; HLA = human leukocyte antigen; MHC = major histocompatibility complex; RA = rheumatoid arthritis; SE = shared epitope.

*0404, were significantly associated with RA, and it was noted that the *0404 allele apparently had the strongest effect, but this point was not formally proven because the relative risk values for these two alleles had overlapping confidence limits. This study also provided evidence that the risk conferred by carrying two SE+ DRB1 alleles was only slightly greater than the risk conferred by carrying one SE+ DRB1 allele, with the exception of SE+ combinations that included the DRB1*0404 allele. However, the latter interpretation was based on relatively few patients and needs confirmation. Future follow-up studies of the Norfolk cohort [5] will most likely provide important information about the possible role of SE+ DRB1 alleles in the clinical course of RA.

HLA-DR or HLA-DQ as the primary risk factor for RA

On the basis of observations of experimental mouse models of collagen-induced arthritis, Zanelli *et al* [6] advanced the provocative hypothesis; that it is HLA-DQ molecules that predispose to RA, whereas DR molecules are either permissive or protective. In the first variant of this hypothesis, it was proposed that it was the DQB1 locus which was responsible for the DQ association. However, a comprehensive review of the literature demonstrated that the DQB1 association with RA is secondary to the HLA-DRB1 association [7]. Zanelli *et al* [8] subsequently introduced a revised version of the DQ-association hypothesis. One of the cornerstones in this hypothesis was the observation that individuals carrying certain HLA-DQA1 alleles (DQA-RA) are highly susceptible to developing RA. Interestingly, these DQA alleles were not investigated in the reported patients or controls, but their presence or absence was deduced from the presence of certain DRB1 and DQB1 alleles. On the basis of these data we have tested the RA-association of their DQA-RA alleles against the RA-association of the SE alleles (see supplementary data below) using our previously described method [9]. It was found that the SE association is still significant when stratified for the DQA1-RA allele combination, while the DQA1-RA combination is not significant when stratified for SE, indicating that the association with SE is stronger than that for the DQA1-RA allele combination. In a subsequent report, Zanelli and coworkers [10] claim to find support for their hypothesis by introducing a new variable: homozygosity for some but not all of their DQA-RA markers. But because these data are to some extent at variance with their earlier ones, support for their hypothesis is not evident and difficult to accept. Taken together, our analyses do not support the idea that HLA-DQ molecules play a major role in the general susceptibility to RA, and demonstrate that the strongest association in RA is with DRB1 genes rather than DQB1 or DQA1 genes. This conclusion is further substantiated by three studies that also found no support for HLA-DQ encoded susceptibility in RA patients from Germany [11], Holland [12] and Australia [13].

HLA class II transgenic mice and RA

The molecular basis for the HLA-DRB1 association with RA is still unclear. One prevailing hypothesis is that the RA-associated HLA-DR molecules present self-antigens to autoaggressive T cells, which subsequently induce an inflammatory response that leads to the development of arthritis. This hypothesis is based partly on the biological role of MHC class II molecules in T cell dependent immunity and the presence of T cells in the synovial compartment, and partly on extrapolated data from other human HLA class II associated autoimmune disorders such as insulin-dependent diabetes mellitus and multiple sclerosis, and the animal models for these diseases. However, the sporadic evidence for the involvement of autoreactive T cells in the pathogenesis of RA [14], probably reflects several competing factors, some of which are related to difficulties in sampling T cells from RA patients. First, autoreactive T cells do not need to be present in large numbers; second, sampling generally occurs considerably after the inflammatory process has started, which excludes the analysis of T cells that are short-lived and/or play a role only in the initiation of the disease process; and third, patients are often on multiple immunomodulatory medications that further complicate sampling and subsequent analysis of T cell reactivity. Furthermore, it is likely that several autoantigens are targeted by inflammatory attacks, and that the relative involvement of these autoantigens may change from patient to patient and within the individual patient as disease progresses [15].

To delineate the role of RA-associated DR molecules in immune responses possibly related to RA in a less complex biological setting than RA patients, transgenic mice expressing DR4 (DRB1*0401) and DR1 (DRB1*0101) were generated [16,17]. It was initially shown that the human class II molecules in the thymus contributed to the selection of the mouse T cell repertoire, and in the peripheral lymphoid compartment mediated T cell responses to different antigens upon immunization. These studies demonstrated, therefore, for the first time, that it is possible to generate functional human MHC class II transgenic mice. Of more direct relevance to RA, it was subsequently shown that these mice were useful for identifying T cell epitopes in proteins such as collagen type II (CII), which is a candidate autoantigen in RA. The dominant DRB1*0401 and DRB1*0101 restricted T cell epitope in CII was shown to correspond to residues 261–273 [18,19], which is interesting because this epitope overlaps with the dominant CII T cell epitope presented by the mouse MHC class II molecule, I-A^q, associated with collagen-induced arthritis (CIA) [20,21]. Moreover, by defining MHC and T cell receptor contacts in CII 261–273 peptide [22] and by

generating a molecular model of the DRB1*0401 molecule in complex with this peptide [23], it was directly demonstrated that the CII 261–273 peptide matches the peptide binding specificity of RA-associated DR molecules [24]. The important question then was whether the CII 261–273 peptide is recognized by T cells from RA patients. Two recent studies have investigated this, and came to different conclusions [25,26].

In the first study [25], T cell proliferative responses to native CII and a CII 255–275 peptide (including the 261–273 peptide) were examined in RA patients, osteoarthritis (OA) patients and healthy controls. All medications were stopped 48 h before study entry. Even though the peripheral blood T cell responses to native CII were modest, the stimulation indices and the fraction of individuals with positive T cell responses were significantly higher in the RA group than in the OA patients and the healthy controls. Comparison of synovial fluid and peripheral blood samples from RA patients showed that T cell responses to native CII and the CII peptide in general were higher in synovial fluid than in peripheral blood, and that there was a good correlation between the T cell responses to CII and the CII peptide. Interestingly, those RA patients with a positive T cell response to native CII often had a shorter disease duration than those with negative responses, and positive T cells responses to CII were significantly enhanced in early disease (<3 years) compared with late disease (>3 years). Whether this correlation also extends to the CII peptide was not investigated, but is likely to be the case because of the observed correlation between T cell reactivity to native CII and the CII peptide.

In the second study, fluorescent, soluble CII 261–273 peptide–DRB1*0401 complexes (tetramers) were used to search for $CD4^+$ T cells in synovial fluid from RA patients [26]. The tetramers were shown to stain DRB1*0401-restricted and CII 261–273-specific T cell hybridomas in a specific manner, but did not stain a detectable fraction of synovial $CD4^+$ T cells. This suggests that the major oligoclonal $CD4^+$ T cell expansion set in joints from this group of RA patients does not recognize the dominant CII epitope. However, this may be partly due to the fact that nearly all patients were on multiple immunomodulatory medications on study entry, and partly due to a rather long disease duration in this RA group (mean duration of disease, 13.8 years; range, 5–28 years). It will be interesting to see whether tetramer stainings of T cells from RA patients with shorter disease duration give another result, and also interesting to compare tetramer staining and functional T cell assays directly.

Development of humanized animal models for RA

One of the goals in generating transgenic mice expressing RA-associated DR molecules was to develop humanized animal models for RA. Neither DRB1*0401 nor DRB1*0101 transgenic mice develop spontaneous arthritis [16,17], which, however, was expected as RA is a polygenic disease with genetic factors other than HLA class II. In addition, undefined nongenetic factors are thought to play an important role in the development of disease [4]. When DRB1*0401 [22,27] and DRB1*0101 transgenic mice [19] are immunized with native CII emulsified in complete Freund's adjuvant, the majority of the animals develop inflammatory arthritis, which has interesting similarities with RA and is more or less indistinguishable from classical CIA as seen in, for example, H-2q mice [28]. Somewhat surprisingly, DRB1*0401 and DRB1*0101 transgenic mice seem to be equally susceptible to CIA, which clearly is in contrast to the situation in humans, where DR4 is a stronger RA-risk gene than DR1. Furthermore, both strains develop severe arthritis, which is also at variance with the risk factor situation for RA, where DRB1*0401 is associated with more severe disease than DRB1*0101 [3]. A trivial explanation for these discrepancies arises from the obvious fact that CIA is a disease provoked by immunization with CII in complete Freund's adjuvant, and thus differs from RA. This powerful arthritis induction scheme may override the differential risks and severities conferred by DRB1*0401 and DRB1*0101 in humans, which may also depend on complex interactions with proteins encoded by other (non-HLA) loci. Such epistatic interactions are most likely difficult to reproduce in transgenic mice expressing a single human disease-risk gene. These comparisons across the species barrier demonstrate that one should be cautious in extrapolating from humanized animal models of disease to actual human diseases.

Another example of how cautious one should be in the interpretation of results from humanized animal models comes from a study on HLA-DQ8 transgenic mice [29]. These mice also develop a severe inflammatory arthritis upon immunization with CII and complete Freund's adjuvant, which merely demonstrates that the DQ8 molecule is permissive for CIA in mice. This observation, together with additional data from nonhumanized animal models of CIA, was taken as evidence for the hypothesis that DQ8 rather than DR4 confers the strongest susceptibility to RA in the DR4-DQ8 haplotype. As already discussed, this hypothesis has very short roots in human genetics, and illustrates that the development of humanized animal models should be based on careful analyses of human genetics.

Conclusion

The development of humanized animal models for RA has so far been shown to be a feasible approach, but, even taken together, the models have added very little to our understanding of this disease. The development of a new generation of humanized models in which the RA-associated HLA class II transgenes are expressed more physiologically, and where additional RA susceptibility genes are incorporated, combined with a better understanding of the

nongenetic component of the disease will provide a more optimal setting for mechanistic studies of the disease process and, ultimately, the development of new drugs.

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Supplementary data

On the basis of Tables 2 and 3 in Zanelli *et al* [8], the RA-association of the DQA-RA marker was tested against that of the SE marker using a 2 × 4 table [9]. This procedure is illustrated here in Tables 1 and 2 on Dutch patient and control data from Zanelli *et al* [8]. It appears that both the DQA-RA and SE markers are quite strongly associated with RA. Stratification of the two markers shows that the DQA-RA marker cannot be tested in SE-positive patients and controls because all SE-positive individuals were also DQA-RA-positive, leaving no information. When testing the DQA-RA association in SE-negative patients and controls, the association is no longer significant. Conversely, when testing the SE association in DQA-RA-positive patients and controls, it appears that this association is still significant, indicating a stronger association with SE than with DQA-RA. Unfortunately, the absolute inclusion of SE in DQA-RA patients prohibits testing of SE in DQA-RA-negative individuals. When combining the data on Dutch people with those on Swiss individuals in [8] in Table 3, the results in Table 1 are further supported: the stratification procedure gives only evidence for a stronger SE than a DQA-RA association, and it may be noted that there is no significant heterogeneity between the two data sets.

Table 1

A 2 × 4 table of DQA-RA and SE markers in Dutch patients with RA and Dutch controls

DQA-RA	SE	Patients	Controls
+	+	172	127
+	–	7	19
–	+	0	0
–	–	58	160
Total		237	306

Data from Zannelli *et al* [8]. DQA-RA, RA-associated DQA markers as defined by Zanelli *et al* [8]; SE, shared epitope for RA-associated HLA-DR markers.

Table 2

The 2 × 2 analyses [9] of the data in Table 1

Test	Comparison	a	b	c	d	Odds ratio	Fisher's *P* value
DQA-RA association		179	58	146	160	3.4	3E-11
SE association		172	65	127	179	3.7	3E-13
DQA-RA association in SE-positive	++ vs –+	172	0	127	0	ND	ND
DQA-RA association in SE-negative	+– vs ––	7	58	19	160	1.1	NS
SE association in DQ-RA-positive	++ vs +–	172	7	127	19	3.5	0.0024; *Pc* = 0.029
SE association in DQ-RA-negative	–+ vs ––	0	58	0	160	ND	ND

ND, Not defined; NS, not significant; *Pc*, corrected *P* value; a and b, numbers of patients with and without marker in question; c and d, numbers of controls with and without marker in question.

Table 3

Combined analyses in 2 × 2 analyses of data in Table 1 and data on Swiss patients and controls given in Zanussi *et al* [8]

Test	Odds ratio	Significance*	*P*	Heterogeneity†	*P*‡
DQA-RA association	3.4	90.5	2E-21	1.35	0.51
SE association	4.1	102.0	5E-24	0.62	0.74
DQA-RA association in SE-positive	ND				
DQA-RA association in SE-negative	1.5	1.7	0.19	1.2	0.54
SE association in DQ-RA-positive	2.9	12.7	0.0004	0.35	0.84
SE association in DQ-RA-negative	ND				

ND, Not defined. * Significance for the deviation of odds ratio from unity. † Heteroengeneity between the two sets of data. ‡ Significance for the heterogeneity.

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Synthetic amino acid copolymers that bind to HLA-DR proteins and inhibit type II collagen-reactive T cell clones

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ABSTRACT **Copolymer 1 [poly(Y,E,A,K)] is a random synthetic amino acid copolymer of L-tyrosine, L-glutamic acid, L-alanine, and L-lysine that is effective both in suppression of experimental allergic encephalomyelitis and in the treatment of relapsing forms of multiple sclerosis. Copolymer 1 binds promiscuously and very efficiently to purified HLA-DR molecules within the peptide-binding groove. In the present study, YEAK and YEAK-related copolymers and type II collagen (CII) peptide 261–273, a candidate autoantigen in rheumatoid arthritis (RA), competed for binding to RA-associated HLA-DR molecules encoded by DRB1*0101 and DRB1*0401. Moreover, these copolymers (particularly YEAK, YAK, and YEK) inhibited the response of DR1- and DR4-restricted T cell clones to the CII epitope 261–273 by >50%. This direct evidence both for competitive interactions of these copolymers and CII peptide with RA-associated HLA-DR molecules and for inhibition of CII-specific T cell responses suggests that these compounds should be evaluated in animal models for rheumatoid arthritis.**

Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of ≈1% among Caucasians (1, 2). It is characterized by a chronic inflammation of the synovial joints and infiltration by activated T cells, macrophages, and plasma cells (3, 4), leading to a progressive destruction of the articular cartilage. Inherited susceptibility to RA is strongly associated with the DRB1 loci encoding HLA-DR1 (DRB1*0101) and -DR4 (DRB1*0401, DRB1*0404, or DRB1*0405) molecules (5–7). Residues 67–71 are polymorphic in HLA-DR proteins, but these RA-related alleles share a common DRβ motif in this region that contributes to the P4 pocket of the peptide-binding groove (8) as well as residues which interact with the T cell receptor of $CD4^+$ T lymphocytes (9–11). It has been proposed that RA-associated HLA-DR molecules confer disease susceptibility by presenting distinct sets of antigenic peptides derived from a synovial joint protein(s) to $CD4^+$ T lymphocytes (12, 13). Although the nature of the autoantigen(s) in RA is unknown, type II collagen (CII) has been proposed as a candidate because it is a major protein of hyaline cartilage and is able to induce arthritis resembling RA in genetically susceptible animals (14–22). Animal models for collagen-induced arthritis, including mice transgenic for HLA-DR1 or -DR4 (21, 22), enabled mapping of T cell determinants implicated in the autoimmune response to CII (23–25). An immunodominant T cell epitope in CII corresponding to residues 261–273 has been identified (24).

Copolymer 1 [Cop 1, poly(Y, E, A, K), called YEAK hereinafter] is a synthetic amino acid copolymer effective both in suppression of experimental allergic encephalomyelitis (26–36) and in the treatment of relapsing forms of multiple sclerosis (37, 38). Recently, the binding of Cop 1 to purified HLA-DR molecules within the peptide-binding groove has been reported (39). Cop 1 inhibited the binding of HA306–318 peptide, a high-affinity epitope of influenza virus, to both HLA-DR1 (DRB1*0101) and -DR4 (DRB1*0401) molecules (39). Copolymers composed of only three amino acids (EAK, YEA, YAK, and YEK) bound to living antigen-presenting cells (APCs) of both mouse and human origin and were cross-reactive with Cop 1 at the T cell level (M.F.-H., R. Aharoni, D. Teitelbaum, R. Arnon, M. Sela, and J.L.S., unpublished observations). In view of the possible therapeutic applications of Cop 1 or related copolymers in RA, it was important to determine whether these compounds compete with CII for binding to HLA-DR1 and -DR4 molecules. In the present report, the competition of Cop 1 and other copolymers with CII261–273 peptide for binding to RA-associated HLA-DR1 and -DR4 molecules was established. Moreover, these copolymers (particularly YEAK, YAK, and YEK) inhibited the response of DR1- and DR4-restricted T cell clones to the CII261–273 epitope. These findings provide direct evidence for competitive interactions between these copolymers and CII peptide for binding to RA-associated HLA-DR molecules and for inhibition of the CII-specific T cell response, suggesting the possible utility of these compounds in the treatment of RA.

MATERIALS AND METHODS

Protein Expression and Purification. Recombinant HLA-DR1 and -DR4 molecules were expressed in *Drosophila* S2 cells as described (11, 40). Cells were grown in roller bottles in ExCell 401 medium (Sigma) supplemented with 0–5% fetal bovine serum (Sigma) at 26°C. Cells were harvested 4–5 days after induction by 1 mM $CuSO_4$. Immunoaffinity purification of recombinant HLA-DR1 and -DR4 molecules was performed as reported (11). Briefly, supernatant from harvested cells was sequentially passed through protein A, protein G, and protein A-LB3.1 columns, followed by elution of the bound HLA-DR with 50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 11.5); next, the supernatant was neutralized with 200 mM phosphate (pH 6.0). Proteins were concentrated on a Centriprep 10 membrane (Amicon).

Peptides and Proteins. Cop 1 (YEAK) is a synthetic random copolymer prepared by polymerization of the *N*-carboxyanhydrides of L-tyrosine, γ-benzyl-L-glutamate, L-alanine, and ε,*N*-trifluoroacetyl-L-lysine (26); the end product is a mixture of acetate salts of random polypeptides. EAK, YEA, YAK, and YEK were synthesized similarly. The following copolymers were synthesized by Teva Pharmaceutical Industries (Petach Tikva, Israel): Cop 1, YEAK, batch 55495 in the molar ratio of 1 Y: 1.5 E: 4.8 A: 3.7 K, with an average molecular weight

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Abbreviations: RA, rheumatoid arthritis; CII, type II collagen; Cop 1, copolymer 1, YEAK; HA, influenza virus hemagglutinin; MW, molecular weight; IL, interleukin; APC, antigen-presenting cell.

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(MW) of 5,800 or batch 52596 in the molar ratio of 1 Y: 1.5 E: 4.3 A: 3.3 K, MW 8,150; EAK, batch SD-1689 in the molar ratio of 1.2 E: 4.3 A: 3 K, MW 8,850; YEA, batch SD-1690 in the molar ratio of 1 Y: 0.6 E: 3 A, MW 7,600; YAK, batch SD-1691 in the molar ratio of 1 Y: 4.3 A: 3.1 K, MW 20,000; and YEK, batch SD-1697 in the molar ratio of 1 Y: 1 E: 3 K, MW 11,050.

Peptides were synthesized by using solid phase techniques (41) on an Applied Biosystems Peptide Synthesizer and were purified by RP-HPLC. Peptide sequences were HA306–318, PKYVKQNTLKLAT (MW 1,718) and CII261–273, AGFKGEQGPKGEP (MW 1,516).

Peptide Labeling. Biotinylation of YEAK, EAK, YEA, YAK, and YEK was performed with excess *N*-hydroxysuccinimide biotin (Sigma) in dimethyl sulfoxide as described (34). Unreacted biotin was removed by dialysis (Spectra/Por membrane MWCO 500, Spectrum Medical Industries).

Assays for Peptide Binding to Class II Major Histocompatibility Complex Proteins. *Solutions.* The solutions used in this assay are the following: binding buffer (20 mM Mes/140 mM NaCl/0.05% NaN_3, pH 5.0) unless otherwise specified, PBS (150 mM sodium chloride/7.5 mM sodium phosphate, dibasic/2.5 mM sodium phosphate, monobasic, pH 7.2), Tris-buffered saline (TBS) (137 mM sodium chloride/25 mM Tris, pH 8.0/2.7 mM potassium chloride); and TBS plus 0.05% Tween 20.

Microtiter assay plate preparation. The 96-well microtiter immunoassay plates (Pro-Bind, Falcon) were coated with 1 μg/well of affinity-purified LB3.1 mAb in PBS (100 μl total) for 18 h at 4°C. The wells were then blocked with TBS/3% BSA for 1 h at 37°C and washed three times with TBS plus 0.05% Tween 20. Before sample addition, 50 μl of TBS/1% BSA was added to each well.

Inhibition reactions. Biotinylated YEAK, YEA, YAK, EAK, or YEK at a final concentration of 1.5 μM in 50 μl of the binding buffer was coincubated with unlabeled inhibitors (YEAK, YEA, YAK, EAK, YEK, CII261–273, or HA306–318) and HLA-DR molecules for 40 h at 37°C.

Detection of class II major histocompatibility complex protein/peptide complexes. Bound peptide–biotin was detected by using streptavidin-conjugated alkaline phosphatase as follows: Plates were washed three times with TBS plus 0.05% Tween 20 and incubated with 100 μl of streptavidin-conjugated alkaline phosphatase (1:3,000, Bio-Rad) for 1 h at 37°C, followed by addition of *p*-nitrophenyl phosphate in triethanolamine buffer (Bio-Rad). The absorbance at 410 nm caused by released *p*-nitrophenyl phosphate was monitored by a microplate reader (Dynatech MR4000).

T Cell Hybridomas and Antigen Presentation Assays. The following mouse T cell hybridomas specific for CII were used: HLA-DR1-restricted 3.19 and 19.3 clones (22) and HLA-DR4-restricted 3838 and D3 clones (25). APCs were L57.23 [L cells transfected with HLA-DR1 (22)], L cells transfected with HLA-DR4 (42), and Priess cells (DRB1*0401/DRB4*0101). T cell stimulation experiments were performed in 96-well microtiter plates in a total volume of 0.2 ml. Irradiated (3,000 rad) APCs (2.5×10^4/well) were coincubated with CII261–273 (40 μg/ml) and varying concentrations of copolymers for 2 h at 37°C; next T cells (5×10^4/well) were added and incubated for 24 h at 37°C. Supernatants (30 μl) were taken and incubated with interleukin 2 (IL-2)-dependent CTLL cells (5×10^4/well) for 12 h, followed by labeling with [^{3}H]thymidine (1 μCi/well) for 12 h. Plates were harvested, and the radioactivity was monitored by using a 1450 Microbeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD).

RESULTS

Inhibition of Binding of Random Synthetic Copolymers to Recombinant HLA-DR1 and -DR4 Molecules by CII261–273 Epitope. Three different preparations of Cop 1 (YEAK) bound to purified HLA-DR1, -DR2, and -DR4 molecules with high affinity and in a peptide-specific manner (39). Recently, copolymers composed of only three amino acids YEA, YEK, YAK, and EAK were also shown to bind these HLA-DR molecules with high affinity and to compete with Cop 1 for binding (M.F.-H., R. Aharoni, D. Teitelbaum, R. Arnon, M. Sela, and J.L.S., unpublished observations). To determine whether YAK, YEK, YEA, or YEAK competed with the RA-associated epitope CII261–273 for binding to HLA-DR1 or -DR4 molecules, recombinant water-soluble HLA-DR1 and -DR4 proteins (encoded by DRA/DRB1*0101 and *0401, respectively) were employed (EAK was excluded from the study because its binding to and competitive efficiency for HLA-DR1 and -DR4 was low). Competitive binding assays were carried out with biotinylated YEAK, YAK, YEA, or YEK and unlabeled inhibitors (YEAK, YAK, YEA, YEK, CII261–273, or HA306–318 peptide) (Fig. 1).The binding of biotinylated YEAK, YEA, or YAK to HLA-DR1 or -DR4 molecules was less well inhibited by the CII261–273 epitope than was the binding of YEK, as expressed by higher IC_{50} values for the CII peptide. HA306–318 inhibited the binding of each copolymer more efficiently than did CII261–273 (Fig. 1 *A* and *B* and Table 1), but the inhibition of binding of the various biotinylated copolymers was in the same order with YAK and YEAK as the best competitors (i.e., higher IC_{50} values for the HA peptide). These experiments indicate that

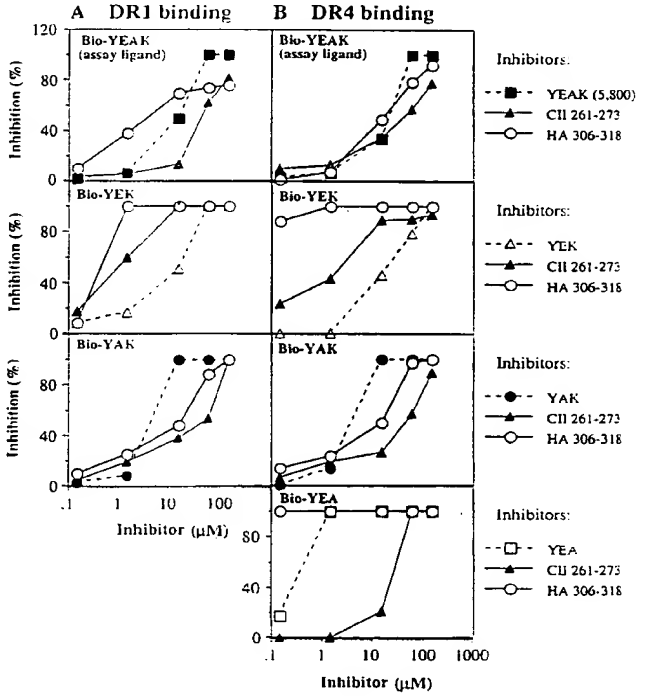

FIG. 1. Inhibition of binding of biotinylated copolymers to recombinant HLA-DR1 (*A*) and -DR4 (*B*) molecules by different competitors. Recombinant water-soluble HLA-DR1 or -DR4 molecules (0.15 μM) were incubated with biotinylated YEAK (MW 5,800), YEK (MW 11,050), YAK (MW 20,000), or YEA (MW 7,600) (1.5 μM) in the presence of unlabeled copolymers, HA306–318, or CII261–273 at a range of concentrations. All incubations were carried out in duplicate at pH 5.0 for 40 h at 37°C. The amount of bound biotinylated copolymer (assay ligand) was measured as described. Specific binding is expressed as the percentage of inhibition by using the following formula: percentage of inhibition = 100% − [(absorbance at 410 nm with competitor − background)/(absorbance without competitor − background) × 100]. The signals at 410 nm without competitor were 0.91–0.95, and the background was 0.17.

Table 1. IC_{50} for inhibition of binding of copolymers to recombinant HLA-DR1 and -DR4 molecules by antigenic peptides

Biotinylated copolymer*	HLA-DR1†		HLA-DR4	
	CII261–273	HA306–318	CII261–273	HA306–318
YEAK	40.0‡	3.5	40.0	10.5
YEK	0.8	0.4	2.0	≪0.1
YAK	40.0	10.5	40.0	10.5
YEA	ND§	ND	23.0	≪0.1

*Recombinant HLA-DR1 and -DR4 molecules were purified as described.

†YEAK with an average MW of 5,800; YAK, MW 20,000; EAK, MW 8,850; YEA, MW 7,600; and YEK, MW 11,050 (1.5 μM) were incubated with CII261–273 or HA306–318 peptides at a range of concentrations and with purified HLA-DR1 and -DR4 molecules at pH 5.0 followed by capture with class II-specific mAb and peptide detection with alkaline phosphatase-streptavidin.

‡Data are presented as IC_{50} (μM, inhibitory concentration of CII or HA peptide giving 50% inhibition of the binding of biotinylated copolymers). These values were calculated based on the competitive binding assays (Fig. 1) (i.e. the higher the IC_{50} of the peptide, the more effective the copolymer in competing for binding of the peptide).

§ND, not determined.

random copolymers compete with and prevent binding of the autoantigenic epitope CII261–273 to RA-associated HLA-DR1 or -DR4 molecules.

Inhibition of HLA-DR1- and -DR4-Restricted CII-Specific T Cell Response by Random Copolymers. To determine whether YAK, YEK, YEA, or YEAK could also inhibit presentation of the CII261–273 peptide to autoreactive T cells, CII-specific T cell hybridomas restricted to HLA-DR1 (3.19 and 19.3) (22) and HLA-DR4 (3838 and D3) (25) were examined. Irradiated APCs were incubated with CII261–273 and relevant copolymer for 2 h before T cells were added for 24 h, and supernatants were tested for IL-2 secretion by these hybridomas. YAK was the most potent inhibitor of HLA-DR1-restricted T cells, by using L fibroblasts transfected with HLA-DR1 as APCs for the CII peptide, whereas the other copolymers inhibited the response less efficiently (Fig. 2). A similar pattern of activity was obtained for HLA-DR4-restricted T cells by using either Priess or L fibroblasts transfected with HLA-DR4 as APCs (Fig. 3 *A* and *B*). Thus, based on these data, the ability of random copolymers to compete with the potential autoantigenic CII261–273 peptide presented to the T cell hybridomas is expressed in the following order: YAK > YEK > YEAK ≫ YEA.

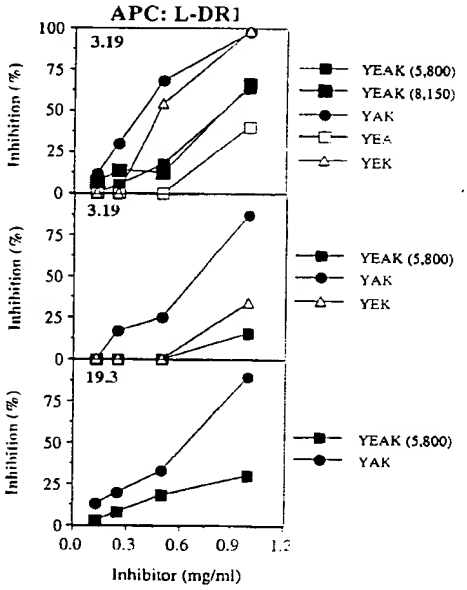

FIG. 2. Inhibition of IL-2 production by HLA-DR1-restricted, CII-specific T cell hybridomas in the presence of different copolymers. Irradiated L57.23 cells were coincubated in duplicate with CII261–273 (40 μg/ml) and varying concentrations of copolymers for 2 h at 37°C; next, T cells (clones 3.19 or 19.3) were added and incubated for 24 h at 37°C. Supernatants (30 μl) were incubated with IL-2-dependent CTL-L as described. Data are presented as the percentage of inhibition of CTL-L proliferation by using the following formula: percentage of inhibition = 1 − [(cpm in the presence of inhibitor − background)/(cpm in the absence of inhibitor − background) × 100].

DISCUSSION

In this report, direct evidence for inhibition of the CII-specific T cell response by 4- and 3-aa random synthetic copolymers is provided, based on competition for binding to RA-associated HLA-DR1 and -DR4 molecules and on decrease in IL-2 production by HLA-DR1- and -DR4-restricted T cell hybridomas in the presence of these copolymers.

Previous findings suggested that the activity of Cop 1 in experimental allergic encephalomyelitis and multiple sclerosis involves binding to class II major histocompatibility complex molecules within the peptide-binding groove, where it may act either as a blocking peptide or as an antagonist or partial agonist, resulting in suppression of autoimmune T cell responses (39). Here, the binding of Cop 1 to RA-associated HLA-DR1 (DRB1*0101) and -DR4 (DRB1*0401) molecules was competed by the peptide determinant CII261–273, a candidate autoantigen in RA. In addition to Cop 1 (YEAK), copolymers of three amino acids, in particular YAK and YEK, which were shown to bind purified HLA-DR1 and -DR4 molecules with high affinity (M.F.-H., R. Aharoni, D. Teitelbaum, R. Arnon, M. Sela, and J.L.S., unpublished observations), competed for binding with CII peptide very efficiently. In these binding experiments, recombinant "empty" HLA-DR1 and -DR4 molecules were employed, with no interference from the endogenous peptides, as opposed to previously analyzed HLA-DR1 and -DR4 molecules, of which only 10–20% are available for exogenous peptide binding (43); this alteration resulted in different binding affinities for the copolymers in these two reports. In addition, the use of empty molecules may account for differences in the affinity of CII261–273 binding in the competitive assays with various copolymers. Previous reports have determined the require-

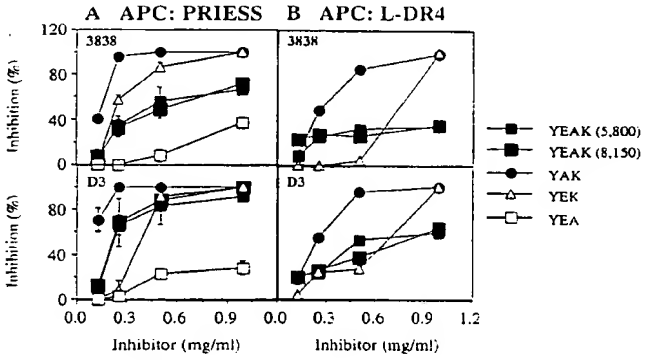

FIG. 3. Inhibition of IL-2 production by HLA-DR4-restricted, CII-specific T cell hybridomas in the presence of different copolymers. Irradiated Priess cells (*A*) or L cells transfected with HLA-DR4 (*B*) were coincubated in duplicate with CII261–273 (40 μg/ml) and varying concentrations of copolymers for 2 h at 37°C; next, T cells (clones 3838 or D3) were added and incubated for 24 h at 37°C. Results represent the mean ± SD of two independent experiments. Other details are described in the legend to Fig. 2.

ments for CII261–273 epitope binding to DRB1*0401 as well as T cell receptor contact residues by using human DR4 purified from Priess cells (24, 25). In these studies, Phe 263 of the CII261–273 peptide was aligned to the P1 pocket and Gln 266 was aligned to the P4 pocket of the HLA-DR4-binding groove, in agreement with peptide-binding requirements for these pockets (44). The core of the T cell determinant was found to be similar for HLA-DR1 and -DR4 alleles (22, 24). Because the P1 pocket of both DR1 and DR4 molecules can accommodate Y, any of the random copolymers (YEAK, YAK, or YEK) would have the potential to block the CII261–273 binding.

The inhibitory concentrations of YAK (0.05–0.7 mg/ml), YEAK (0.2–0.85 mg/ml), and YEK (0.23–0.73 mg/ml) that reduced the secretion of IL-2 by HLA-DR1- and -DR4-restricted T cell hybridomas (IC_{50}), were higher than those of different defined antigenic peptides usually used for inhibition of binding to major histocompatibility complex proteins (45–47). However, these concentrations were within the range reported to inhibit both myelin basic protein-specific (32, 33) or proteolipid protein-specific (35) T cell lines and clones by Cop 1 (YEAK) and antigen-specific T cell activation by different random copolymers (48) (0.1–0.5 mg/ml). This may be caused by the fact that these copolymers are mixtures of random polypeptides; consequently, each component is underrepresented in the mixture, requiring higher molar amounts to obtain the inhibitory effect. Further studies, particularly *in vivo* studies in murine arthritis models, will indicate whether any of these compounds are potentially useful in the treatment of RA in humans.

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Class II MHC quantitative binding motifs derived from a large molecular database with a versatile iterative stepwise discriminant analysis meta-algorithm

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Abstract

Motivation: *The identification of T-cell epitopes can be crucial for vaccine development. An epitope is a peptide segment that binds to both a T-cell receptor and a major histocompatibility complex (MHC) molecule. Predicting which peptide segments bind MHC molecules is the first step in epitope prediction.*

Results: *An iterative stepwise discriminant analysis meta-algorithm explores a large molecular database to derive quantitative motifs for peptide binding. The applications presented here demonstrate the algorithm's versatility by producing four closely related models for HLA-DR1. Two models use an expert initial estimate and two do not; two models use amino acid residues as the only predictors and two use amino acid groupings as additional predictors. Each model correctly classifies >90% of the peptides in the database.*

Availability: *Software is available commercially; data are free over the Internet.*

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Introduction

Class II major histocompatibility complex (MHC) molecules play an essential role in the body's immune response to pathogens. Once an antigen-presenting cell (macrophage, dendritic cell or B lymphocyte) captures an extracellular pathogen, the protein component is degraded into peptide fragments. Class II MHC molecules in the cell's interior bind to selected peptide fragments designated antigenic peptides or epitopes. The epitope/MHC complex travels to the cell surface where the MHC molecule displays the epitope to nearby CD4 T lymphocytes. When a CD4 T lymphocyte binds to an epitope/MHC complex, an immune response is initiated.

Each binding peptide is comprised of a linear arrangement of amino acid residues. Knowledge of the amino acid sequence of an epitope is useful in vaccine development and increases general understanding of the immune system. Finding a pattern or motif among epitopes that bind a particular MHC molecule allows for epitope prediction. A predictive model can be used to screen large numbers of potential binding peptides, thus reducing laboratory time and costs.

The challenge of predicting peptide/MHC binding involves numerous molecules and peptides. HLA, the human MHC molecule, is highly polymorphic. Over 200 types of HLA class I and class II molecules have been identified; however, many have been shown to share overlapping binding repertoires (Southwood *et al.*, 1998). Each position in a peptide fragment is occupied by one of 20 amino acid residues. Thus, a peptide of length n has 20^n possible configurations.

The class II MHC binding site has been shown to bind ligands of 9–25 residues. X-ray crystallography reveals that the binding site is open at both ends (Brown *et al.*, 1993; Stern *et al.*, 1994). Consequently, given a class II MHC binding peptide, it is not known which segment is involved in the binding. An algorithm for motif derivation must involve proper alignment as well as motif extraction.

O'Sullivan *et al.* (1991a,b), Chicz *et al.* (1992), Kropshofer *et al.* (1992), Hammer *et al.* (1992, 1993), Falk *et al.* (1994), Fleckenstein *et al.* (1996) and Southwood *et al.* (1998) have studied the binding properties of the class II MHC allele HLA-DR1. In previous studies (Mallios, 1997, 1998), the present author introduced a data-driven procedure for predicting class II MHC binding with or without the use of an initial suggested motif.

This study utilizes a large Internet database. The four models developed for HLA-DR1 demonstrate the algorithm's versatility. Models can be developed with or without an expert initial estimate. The set of predictors can be limited to individual amino acid residues or can be extended to include residue groupings and other amino acid attributes.

Systems and methods

MHCPEP database

The MHCPEP database (Brusic *et al.*, 1997) is a source for peptides known to bind MHC molecules. It is located on the Internet at http://wehih.wehi.edu.au/mhcpep/. The description reads: 'MHCPEP is a curated database comprising over 13000 peptide sequences known to bind MHC molecules. Entries are compiled from published reports as well as from direct submissions of experimental data. Each entry contains the peptide sequence, its MHC specificity and, when available, experimental method, observed activity, binding affinity, source protein, anchor positions, and publication references.' The 526 peptides that bind DR1 were downloaded for analysis in this study.

A database published by O'Sullivan *et al.* (1990) provides sets of non-binding peptides for DR1, DR2, DR5 and DR52a. Ninety-eight peptides that do not bind DR1 were obtained from this source.

The data-sets

The models developed here explore subsequences of length nine. Nine was selected for modeling DR1 because (i) many previous studies suggest motifs of length nine and (ii) the shortest sequences in the MHCPEP database that bind DR1 consist of nine amino acid residues.

The unique strategy employed here depends on building two working data-sets of subsequences: one set of probable binders and one set of probable non-binders. The non-binding data-set remains constant throughout the process. Based on the assumption that all subsequences of a non-binding peptide are themselves non-binding, the non-binding data-set contains all subsequences of length n, of all non-binding peptides.

For example, HEL 91–106 (SVNCAKKIVSDGDGMN) does not bind DR1. Thus, for $n = 9$, the subsequences SVNCAKKIV, VNCAKKIVS, NCAKKIVSD, CAKKIVSDG, AKKIVSDGD, KKIVSDGDG, KIVSDGDGM and IVSDGDGMN are all entered into the non-binding data-set. All duplicate subsequences are deleted from the non-binding data-set. The 98 non-binding peptides produce 676 unique subsequences of length nine.

The binding data-set, on the other hand, is dynamic and changes from iteration to iteration. It is hoped that each successive iteration selects subsequences from the binding sequences that more accurately reflect the true binding motif. The initial binding data-set can be constructed on the basis of a suggested binding motif or reflect the entire database.

The initial binding data-set

The P1–P6 anchor motif utilized by Southwood *et al.* (1998) provides the initial estimate for the first model. A point is scored for Y, F, W, L, I, V or M in position 1, and S, T, C, A, P, V, I, L or M in position 6. For each binding peptide, the subsequence with the highest score is entered into the initial binding data-set. If the highest score is shared by more than one subsequence, they are all entered.

For example, the DR1 binder hemagglutinin 306–318 (PKYVKQNTLKLAT) has five subsequences of length nine. The subsequences and their scores are PKYVKQNTL (0), KYVKQNTLK (0), YVKQNTLKL (2), VKQNTLKLA (2) and KQNTLKLAT (0). Thus, YVKQNTLKL and VKQNTLKLA are both entered into the initial binding data-set. All duplicate subsequences are deleted from the initial binding data-set.

For the second model, the initial binding data-set is constructed without a suggested motif. In this method, similar to building the non-binding data-set, every subsequence from every binding peptide is entered into the initial binding data-set. Thus, for the binding peptide PKYVKQNTLKLAT, all five subsequences PKYVKQNTL, KYVKQNTLK, YVKQNTLKL, VKQNTLKLA and KQNTLKLAT enter the initial binding data-set. Again, duplicate subsequences are deleted from the initial binding data-set. In addition, all subsequences occurring in both the initial binding data-set and the non-binding data-set are deleted from the initial binding data-set.

Stepwise discriminant analysis

Given two mutually exclusive sets, stepwise discriminant analysis (SDA) (Dixon *et al.*, 1990) builds a Bayesian discriminant function that classifies each element into one of the two sets. Specifically, an element is assigned to a set if the Bayesian posterior probability of belonging to that set exceeds the probability of belonging to the complementary set. Arguments for the function are selected from a list of potential predictor variables.

At Step 0, an F statistic from a one-way analysis of variance is computed for each potential predictor variable to estimate which variable will most accurately separate the sets. The variable with the highest F-value is entered into the discriminant function. In a stepwise manner, additional variables are entered into the discriminant function until the F-values of all remaining variables are below a given minimum. In this study, the minimum F-value required for model entrance is set at 3.5.

The jack-knife method of cross-validation (Afif and Clark, 1990) is used. It is a special case of the general cross-validation method in which the classification functions are computed on a subset of cases, and the probability of misclassification is estimated from the remaining cases. In the jack-knife method, the first case is set aside while a classification function is computed on all remaining cases. The first case is evaluated by the classification function and tallied as being correctly or incorrectly

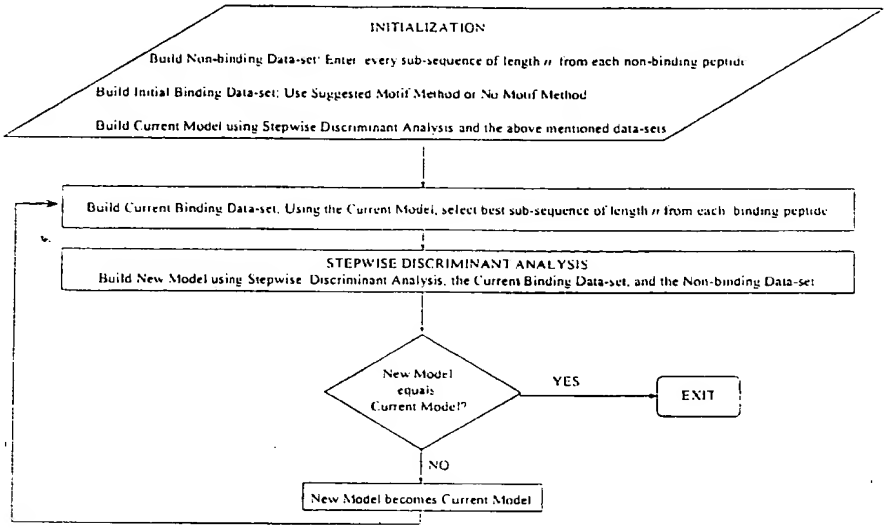

Fig. 1. The iterative algorithm.

classified. The process continues with the second case until each case has been left out in turn and classified.

In this study, the two mutually exclusive sets are the binding data-set of subsequences and the non-binding data-set of subsequences. For the purposes of SDA, elements of the binding data-set are assigned an outcome categorical value of 1 and elements of the non-binding data-set are assigned an outcome categorical value of 0.

The potential predictor variables for each case (subsequence) describe the biochemistry and position of each amino acid residue. If YVKQNTLKL is the subsequence of PKYVKQNTLKLAT that binds DR1, the binding can be depicted by:

```
123456789
YVKQNTLKL
```

Predictor variables A1, C1, ..., Y1 refer to amino acid residues in position 1; A2, C2, ..., Y2 refer to residues in position 2, etc. The single-letter abbreviations for amino acid residues are ordered alphabetically. For a given case (subsequence), potential predictor variables are assigned a 1 if the designated residue occupies the position in question, a 0 if it does not.

For the current example, YVKQNTLKL, Y1 = V2 = K3 = Q4 = N5 = T6 = L7 = K8 = L9 = 1, while A1 ... W1 = C2 ... T2 = W2 ... Y2 = A3 ... I3 = L3... Y3 = A4 ... P4 = R4 ... Y4 = A5 ... M5 = P5 ... Y5 = A6 ... S6 = V6 ... Y6 = A7 ... K7 = M7 ... Y7 = A8 ... I8 = L8 ... Y8 = A9 ... K9 = M9 ... Y9 = 0.

When SDA is performed on the database of outcome variables and associated predictor variables, the result is a mathematical classification function of the form:

$$u = b_0 + b_1 v_1 + b_2 v_2 + \ldots + b_i v_i$$

where i is the number of steps, v_1 through v_i are the predictor variables selected by SDA, and b_0 through b_i are coefficients determined by SDA. Subsequences in the binding data-set will generally have large values of u, while subsequences in the non-binding data-set will generally have small values of u.

A convenient aspect of SDA is that the classification function can be converted into the probability of set membership. The probability (P) that a subsequence belongs to the binding set is determined by the following equation:

$$P = 1/(1 + e^{-u})$$

The decision rule for classification is based on the probability of set membership. Most commonly, a value of $P > 0.5$ is utilized.

The iterative algorithm

A diagram of the iterative algorithm is shown in Figure 1. It is first necessary to select a sequence length, n, as there will be a different resulting model for each value of n. The initial binding and non-binding data-sets are filled with subsequences of length n. SDA is performed utilizing the outcome and predictor variables defined above. While the non-binding data-set remains constant, the resulting SDA classification function is used to select the best subsequences for the next binding data-set. SDA is applied again and the process is repeated until the new classification function is the same as the previous function.

Algorithm implementation and results

For the model using the P1–P6 anchor motif as an initial estimate, the first application of SDA yields the following mathematical classification function which becomes the current model in the algorithm diagram:

u = −5.78 + 1.14 A1 + 3.90 F1 + 3.63 I1 + 3.89 L1 + 3.85 M1 + 0.87 P1 + 4.62 Y1 + 3.85 W1 + 4.22 Y1 − 1.87 C2 − 1.08 E2 − 0.99 G2 − 1.50 H2 − 1.36 P2 − 0.87 S2 + 1.64 W2 + 1.18 A3 + 2.10 F3 + 0.93 K3 + 1.73 L3 + 1.08 M3 + 1.06 Q3 + 0.94 R3 + 2.47 Y3 + 1.56 A4 + 1.35 L4 + 2.02 M4 + 1.76 Q4 − 1.27 T4 + 0.93 V4 − 2.80 W4 + 1.20 L5 + 1.89 M5 + 3.08 A6 + 1.82 C6 − 1.57 E6 + 2.87 I6 + 3.54 L6 + 3.39 M6 + 1.77 P6 + 2.39 S6 + 2.00 T6 + 3.02 V6 − 1.21 D7 − 1.26 E7 − 1.02 K7 − 1.20 N7 + 1.86 F8 + 1.16 I8 + 1.17 L8 + 1.49 V8 + 1.45 A9 + 1.75 F9 + 1.63 I9 + 1.86 L9 + 1.19 Q9 + 1.16 R9 + 2.08 V9

A direct interpretation of this model would be as follows: DR1 binding is strongly encouraged by F, I, L, M, V, W, Y in position 1, and A, L, M, V in position 6. The influence of the P1–P6 motif is apparent. Since the predictor variables are either 1 or 0 (present or absent), the classification function can be replaced by an alignment matrix. Henceforth, results will be reported in matrix form where matrix elements are weights to be added to calculate the binding score.

Applying this SDA classification function to the five subsequences of PKYVKQNTLKLAT yields the following:

u(**PKYVKQNTL**) = −5.78 + 0.87 + 2.47 + 0.93 − 1.20 + 1.86 = −0.85

u(KYVKQNTLK) = −5.78 + 1.17 = − 4.61

u(**YVKQNTLKL**) = −5.78 + 4.22 + 0.93 + 1.76 + 2.00 + 1.86 = 4.99

u(**VKQNTLKLA**) = −5.78 + 4.62 + 1.06 + 3.54 − 1.02 + 1.17 + 1.45 = 5.14

u(KQNTLKLAT) = −5.78 − 1.27 + 1.20 = −5.85

Since u(**VKQNTLKLA**) is the largest, VKQNTLKLA is entered into the current binding data-set. The current binding data-set is complete when this selection process has been repeated for all binding sequences. A new model is built using SDA, the current binding data-set and the stable non-binding data-set. The entire process is repeated until termination at the twelfth iteration, when the resulting new model is identical to the model of the eleventh iteration. The models are equal because the selected subsequences of the binding peptides have stabilized.

Table 1. Final DR1 classification model using the P1–P6 anchor motif as the basis for selecting the initial binding data-set. Coefficients converged on the eleventh iteration

Residue	Position								
con = −10.20	1	2	3	4	5	6	7	8	9
A		1.52	1.65	3.23		1.56		1.78	2.88
C									
D									
E						−2.90	−2.13		
F	6.18	2.56	3.47					2.78	
G					−1.29		1.13		
H									
I	2.75					−3.33			5.39
K		2.38	1.41			−2.71			
L	1.98		3.54	2.45	1.54			2.29	5.33
M	5.57			3.77	6.47	2.44			
N								−1.81	
P									
Q				3.76					1.59
R		2.70	1.42			−2.45		1.30	2.10
S						1.17			
T				−1.93					
V	8.86	1.58					3.95		
W	4.77	5.62		−3.54					−3.23
Y	7.52	2.19	4.43		1.98				

Table 2. Final DR1 classification model using no prior information to select the initial binding data-set. Coefficients converged on the fifteenth iteration

Residue	Position								
con = −10.87	1	2	3	4	5	6	7	8	9
A		4.77		2.86	1.42			2.53	3.09
C									
D	−1.70								
E	−1.53					−2.59	−2.27		
F	4.26	4.94	4.10						−2.55
G					−1.20		2.06		
H									
I					2.79				10.81
K		2.67				−3.55			
L		1.50	1.47	2.28	1.78			2.19	8.27
M				6.10	5.19	3.93		2.29	
N								−1.58	
P					1.13				
Q		2.37		2.90				1.97	
R		2.46				−2.67		2.00	1.63
S				2.25			−1.52		
T									
V	3.22	3.32		1.40	2.53			4.55	4.70
W	4.68	4.17							
Y	5.46	3.78	6.26	−2.48	2.35				

Table 3. Performance of final DR1 classification models utilizing amino acid residues as predictors. Non-binders are classified by subsequence in order to evaluate validity

Initial estimate	Classification method	Sensitivity	Specificity	Accuracy
P1–P6 anchor motif	Final model	96.8% (509/526)	95.3% (644/676)	95.9% (1153/1202)
	Jack-knife	95.1% (500/526)	93.8% (634/676)	94.3% (1134/1202)
No motif	Final model	97.3% (512/526)	95.4% (645/676)	96.3% (1157/1202)
	Jack-knife	96.6% (508/526)	93.9% (635/676)	95.1% (1143/1202)

Table 1 presents the final classification model using the P1–P6 anchor motif as the initial estimate, while Table 2 shows results using no initial motif. Two tables are presented to summarize the classification performance of the models. Both tables use the decision rule that $P > 0.5$ predicts that a subsequence will bind DR1. The results in Table 3 are taken directly from the computer output. Non-binders are reported as subsequences and jack-knife validation classifications are presented. Table 4 reports non-binders as peptides. The specificity and accuracy that are reported in Table 4 more realistically represent the true nature of the problem under investigation. However, there are no jack-knife results available for the peptide scenario because the computer program reports only summary results for the jack-knife procedure. As such, it is not possible to determine whether two missclassified subsequences belong to the same peptide or to different peptides. Thus, Table 3 evaluates the validity of the models, and Table 4 evaluates specificity and accuracy.

The suggested motif method and no motif method generate models with similar predictors (F1, V1, W1, Y1, A2, F2, K2, R2, V2, W2, Y2, F3, L3, Y3, A4, L4, M4, Q4, –G5, L5, M5, Y5, –E6, –K6, M6, –R6, –E7, G7, A8, L8, –N8, R8, A9, I9, L9 and R9) and similar performance. The fact that the two methods appear to converge suggests that the no motif method yields a realistic model.

Extending the list of possible predictors

The list of possible predictors can be augmented with dichotomous and continuous variables that characterize attributes of amino acid residues. Of the many schemes for grouping amino acids, the following was chosen for the purpose of illustration:

ACIDic = { D, E };
ALIPhatic = { A, G, I, L, P, V };
AMIDic = { N, Q };
AROMatic = { F, W,Y };
BASIc = { R, H, K };
HYDRoxylic = { S, T } and
SULFur containing = { C, M }.

It is not necessary that sets be mutually exclusive and collectively exhaustive as these are. Similar to the original set of predictor variables, ACID1, ALIP1, ..., SULF1 refer to amino acid residues in position 1; ACID2, ALIP2, ..., SULF2 refer to amino acid residues in position 2; etc. Potential predictor variables are assigned a 1 if a member of the named set occupies the position in question, a 0 if it does not. For the example YVKQNTLKL, AROM1 = ALIP2 = BASI3 = AMID4 = AMID5 = HYDR6 = ALIP7 = BASI8 = ALIP9 = 1. All other potential predictor variables in the extended list are set to 0.

Two new models for DR1 binding are developed using this extended list of predictors—the first utilizing the P1–P6 anchor motif, the second with no initial estimate. Table 5 and 6 present the final classification models, while Table 7 and 8 summarize the classification performance of both models along with jack-knife validation classifications.

Both models share the following characterization: Position 1 prefers AROMatic residues and V. Positions 2 and 3 are also AROMatic. Position 4 prefers A, L, M and Q, while position 5 accommodates M and Y, but not G. Position 9 prefers ALIPhatic residues. The fact that the negative coefficient for P has the same magnitude as the ALIPhatic coefficient suggests that ALIPhatic might be redefined without P.

This same characterization applies to the first two models produced without the extended predictor list. Since all four models classify the data equally well, it is left to future research to determine the optimal variation.

Table 4. Performance of final DR1 classification models utilizing amino acid residues as predictors. Non-binders are classified by peptide in order to evaluate specificity and accuracy

Initial estimate	Classification method	Sensitivity	Specificity	Accuracy
P1–P6 anchor motif	Final model	96.8% (509/526)	73.5% (72/98)	93.1% (581/624)
No motif	Final model	97.3% (512/526)	75.5% (74/98)	93.9% (586/624)

Table 5. Final DR1 classification model using the P1–P6 anchor motif as the basis for selecting the initial binding data-set and the extended list of possible predictors. Coefficients converged on the thirteenth iteration

Residue	Position								
con = −8.97	1	2	3	4	5	6	7	8	9
A				3.58		1.20			
C			−3.89						
D									1.52
E						−2.26			
F								2.82	
G					−1.31				−3.57
H		−2.73							
I	2.29					−3.03			
K									
L	3.26			4.09					
M	5.60			4.75	5.43				
N							−3.44		
P		−1.58	−1.84					−1.52	−4.70
Q				4.95					
R									
S									
T					2.23				
V	7.56		−2.97				2.79		
W		4.43		−3.52			−3.00		
Y					2.34				
ACID			−0.97			−1.49	−1.47		
ALIP		1.49						1.03	4.21
AMID			−1.19				1.49	−1.22	2.13
AROM	7.03	2.64	1.53						
BASI		2.69				−2.46			
HYDR			−1.70						
SULF									2.07

Table 6. Final DR1 classification model using no prior information to selecting the initial binding data-set and the extended list of possible predictors. Coefficients converged on the thirteenth iteration

Residue	Position								
con = −10.27	1	2	3	4	5	6	7	8	9
A		4.18		3.06				1.99	−4.50
C									
D									
E						−2.91	−2.01		
F						2.40			
G					−2.67		2.38		−8.76
H									
I									
K		2.68				−3.35	1.80		
L			1.99	2.46				2.64	
M				6.15	4.61	2.72		2.55	
N								−4.11	
P									−8.89
Q		1.75		2.84					
R		1.68		1.94		−2.57		2.06	2.07
S				2.35			−1.20		
T									
V	6.50							4.44	−4.14
W			−5.40						
Y					2.39				
ACID	−1.52	−1.47							
ALIP					1.24				8.33
AMID				1.48				2.06	
AROM	5.18	3.89	4.05						−1.43
BASI					−1.26				
HYDR									
SULF									

Table 7. Performance of final DR1 classification models utilizing the extended list of possible predictors. Non-binders are classified by subsequence in order to evaluate validity

Initial estimate	Classification method	Sensitivity	Specificity	Accuracy
P1–P6 anchor motif	Final model	97.1% (511/526)	94.7% (640/676)	95.8% (1151/1202)
	Jack-knife	96.4% (507/526)	93.6% (633/676)	94.8% (1140/1202)
No motif	Final model	97.9% (515/526)	95.1% (643/676)	96.3% (1158/1202)
	Jack-knife	96.8% (509/526)	92.9% (628/676)	94.6% (1137/1202)

Table 8. Performance of final DR1 classification models utilizing the extended list of possible predictors. Non-binders are classified by peptide in order to evaluate specificity and accuracy

Initial estimate	Classification method	Sensitivity	Specificity	Accuracy
P1–P6 anchor motif	Final model	97.1% (511/526)	74.5% (73/98)	93.6% (584/624)
No motif	Final model	97.9% (515/526)	75.5% (74/98)	94.4% (589/624)

Discussion

It is difficult to find studies with which to compare. Most studies evaluate a motif only on binding peptides and do not test the ability of the motif to identify non-binding peptides. O'Sullivan *et al.* (1991b) proposed the following DR1 binding motif: W,F,Y,V,I,L in position 1; A,V,I,L,P,C,S,T in position 6; A,V,I,L,C,S,T,M,Y in position 9. They reported that the motif was present in 69% of good binders, 55% of intermediate binders, 31% of weak binders and 16.5% of negative binders.

Using an evolutionary algorithm and artificial neural network, Brusic *et al.* (1998) predicted HLA-DR4 binding. In

binary classification, they correctly classified 100% of high-affinity binders, 82% of moderate-affinity binders, 30% of low affinity binders and 70% of non-binders. Southwood *et al.* (1998) experimentally developed a motif for DR4 binding. When tested on an independent set of 50 peptides, a conservative threshold resulted in sensitivity = 50.0%, specificity = 94.4% and accuracy = 82.0%, while a lower threshold resulted in sensitivity = 78.6%, specificity = 80.6% and accuracy = 80.0%.

The results of the present study compare favorably with these previous studies. Thus, the iterative SDA meta-algorithm is a promising tool for analyzing class II MHC molecular databases. This approach has the following advantages:

- The resultant model is quantitative and easy to interpret.
- The resultant model can be used to predict peptide binding.
- The approach works with or without a suggested binding motif.
- The approach is versatile and can include dichotomous or continuous properties of amino acid residues as possible predictors.
- Classification results compare favorably with previous studies.

As with any data-driven algorithm, the results are data dependent and change when the data-sets change. However, as the data-sets grow, the sample space is better represented and the influence of individual peptides decreases.

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EXHIBIT 4

Autoimmune Diseases and Models

Disease	Model	Commments	Reference
MS	1) EAE suppression 2) Inhibition of MBP-induced T cell proliferation 3) Recognition by COP-1 specific T cells 4) Cross reaction with anti-COP-1 antibodies		1) U.S. Serial No. 09/768,872 specification, Example 3, pg 30 2) U.S. Serial No. 09/768,872 specification, Example 6, pg 43 3) U.S. Serial No. 09/768,872 specification, Example 7, pg 46 4) U.S. Serial No. 09/768,872 specification, Example 8, pg 48
autoimmune hemolytic anemia			
autoimmune oophoritis			
autoimmune thyroiditis		HLA-DR3	Wan, et al., Hum. Immunol. 2002, Apr, 63(4):301-10
autoimmune uveoretinitis		HLA-DR4	Giuseppina, et al., The Journal of Clinical Investigation, April 2003, Vol. 111, No. 8, 1171-80
chronic immune thrombocytopenic purpura			
colitis		HLA-DR4	Kobayashi, et al., Clin Exp Immunol. 1990 Jun;80(3):400-3

Disease	Model	Commments	Reference
contact sensitivity disease			
diabetes mellitus		HLA-DR4	http://ntri.tamuk.edu/immunology/autoimmunity.html
Graves disease		HLA-DR4	Sridama, et al., *Arch Intern Med.* 1987;147:229-231.
Guillain-Barre's syndrome			
Hashimoto's disease		HLA-DR4	Lymberi, et al., Arch Hellen Med, 16(4), July-August 1999, 337-351
idiopathic myxedema			
myasthenia gravis	Inhibition of T Cells responsive to a Myasthenia Gravis Antigenic peptide	HLA-DR3	U.S. Serial No. 09/768,872 specification, Example 10 pg 56 (COP-1 only) Giraud, et al., Neurology, 2001 57(9): 1555-60
psoriasis,		HLA-DR4	Fatma, et al., Swiss Med Wkly, 2003, 133: 541-543
pemphigus vulgaris		HLA-DR4	Lombardi, et al., J Invest Dermatol. 1999 Jul;113(1):107-10.

Autoimmune Diseases and Models (Cont.)

Disease	Model	Commments	Reference
rheumatoid arthritis	1) Binding to Purified Human Leukocyte Antigens (HLA) 2) Competition with collagen for binding to HLA and inhibition of collagen specific T cell response	HLA-DR 1&4	1) U.S. Serial No. 09/768,872 specification, Example 5, pg 37 Fugger, et al., Arthritis Research, 2000, 2: 208-211. 2) U.S. Serial No. 09/768,872 specification, Pg 17, ln 34 to pg 18 ln 1 and Example 9, pg 52. "Autoimmune Disease Research" http://www.immunotolerance.org/research/autoimmunie/ Fridkis-Hareli, PNAS, 1998, Vol. 95, pp 12528-31
systemic lupus erythematosus	Binding to Purified Human Leukocyte Antigens (HLA)	HLA-DR4	U.S. Serial No. 09/768,872 specification, Example 5, pg 37, Batchelor, et al. Lancet 1980 1(8178):1107-9

1: Hum Immunol. 2002 Apr;63(4):301-10.

HLA-DR and HLA-DQ polymorphism in human thyroglobulin-induced autoimmune thyroiditis: DR3 and DQ8 transgenic mice are susceptible.

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In contrast to H2-based susceptibility to experimental autoimmune thyroiditis (EAT) induced with thyroglobulin (Tg), human leukocyte antigen (HLA) association with Hashimoto's thyroiditis, the human counterpart, is less clear. and determining association is further complicated by DR/DQ linkage disequilibrium. Previously, we addressed the controversial implication of HLA-DR genes by introducing HLA-DRA/DRB1*0301 (DR3) transgene int endogenous class II negative H2Ab(0) mice. EAT induction with either human (h) or mouse (m) Tg demonstrated the permissiveness of DR3 molecules for shared Tg epitopes. Here, we examined the participation of HLA-DQ genes by introducing DQA1*0301/DQB1*0302 (DQ8) transgene into class II negative Ab(0) or class I and II negative beta(2)m((-/-)) Ab(0) mice. About 50% and 80% of HLA-DQ8(+) Ab(0) and beta(2)m(-) Ab(0) mice, respectively, developed moderate EAT after hTg immunization, but only minimal response to mTg. The hTg presentation to hTg-primed cells wa blocked by anti-DQ mAb in vitro. By contrast, HLA-DRB1*1502 (DR2) and *0401 (DR4) transgenes contributed little to hTg induction. Similarly, DQA1*0103/DQB1*0601 or DQA1*0103/DQB1*0602 (DQ6) transgenic A (0) mice were unresponsive to hTg induction and carried no detectable influence in DQ8/DQ6 double transgenic mice. Thus, both HLA-DR and -D polymorphism exists for hTg in autoimmune thyroiditis. The use of defined single or double transgenic mice obviates the complications seen in polygeni human studies.

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A humanized model of experimental autoimmune uveitis in HLA class II transgenic mice

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Experimental autoimmune uveitis (EAU) is a disease of the neural retina induced by immunization with retinal antigens, such as interphotoreceptor retinoid-binding protein (IRBP) and arrestin (retinal soluble antigen, S-Ag). EAU serves as a model for human autoimmune uveitic diseases associated with major histocompatibility complex (HLA) genes, in which patients exhibit immunological responses to retinal antigens. Here we report the development of a humanized EAU model in HLA transgenic (TG) mice. HLA-DR3, -DR4, -DQ6, and -DQ8 TG mice were susceptible to IRBP-induced EAU. Importantly, HLA-DR3 TG mice developed severe EAU with S-Ag, to which wild-type mice are highly resistant. Lymphocyte proliferation was blocked by anti-HLA antibodies, confirming that antigen is functionally presented by the human MHC molecules. Disease could be transferred by immune cells with a Th1-like cytokine profile. Antigen-specific T cell repertoire, as manifested by responses to overlapping peptides derived from S-Ag or IRBP, differed from that of wild-type mice. Interestingly, DR3 TG mice, but not wild-type mice, recognized an immunodominant S-Ag epitope between residues 291 and 310 that overlaps with a region of S-Ag recognized by uveitis patients. Thus, EAU in HLA TG mice offers a new model of uveitis that should represent human disease more faithfully than currently existing models.

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Introduction

Experimental autoimmune uveitis (EAU) is a T cell–mediated autoimmune disease model that targets the neural retina and related tissues. EAU is induced by immunization with preparations of purified retinal antigens or their fragments, of which the retinal soluble antigen (S-Ag, also known as arrestin) and the interphotoreceptor retinoid-binding protein (IRBP) are the best known. This experimental model is used to represent a series of human inflammatory diseases collectively known as uveitis, which are characterized by a frequent presence of immune response to retinal antigens, particularly S-Ag. EAU can also be induced by adoptive transfer of T cells from immunized recipients to naive, genetically compatible hosts (1). In most cases, the pathogenesis of the disease implicates a Th1-type cytokine response (1).

Genetic studies have documented association between the presence or absence of certain HLA alleles and susceptibility to particular autoimmune disorders (2). Autoimmune uveitis is a spectrum of diseases that show differences in clinical manifestations and course, and differences in associations with specific HLA loci. The associated class I or class II alleles may also depend on the ethnic origin of the population studied. Among the class II–associated uveitic syndromes, the *HLA-DRB1*0405* allele, encoding for a variant of the HLA-DR4 antigen, was found to be significantly increased in a Japanese population of Vogt-Koyanagi-Harada patients (3), and this was also confirmed in other populations (4, 5). Clinically similar to Vogt-Koyanagi-Harada syndrome, sympathetic ophthalmia is also associated with HLA-DR4 subtypes in Japanese, British, and Irish populations (6, 7). Intermediate uveitis not associated with multiple sclerosis was associated with the HLA-DR3 antigen,

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Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: experimental autoimmune uveitis (EAU); retinal soluble antigen (S-Ag); interphotoreceptor retinoid-binding protein (IRBP); transgenic (TG); bovine IRBP (bIRBP); bovine retinal soluble antigen (bS-Ag).

while panuveitis showed association with HLA-DR4 in an Italian population (8). Association with HLA-DQ alleles was also described (3, 7, 9), but the strong linkage disequilibrium between some DR and DQ alleles makes it difficult to distinguish whether there was a prevalent role of one molecule or whether the DR and DQ genes acted in an epistatic manner. Genetic associations in uveitis have recently been reviewed in depth (10).

The antigen or antigens that might be involved in the etiology of human uveitis have not been elucidated. Although uveitis patients frequently display cellular and humoral responses to the retinal antigens that are uveitogenic in animals, it is not known whether these responses are causally involved in their disease or represent an epiphenomenon (11). This information is key for future development of antigen-specific therapies for these blinding autoimmune diseases. If indeed these responses are causally related, the immunodominant epitopes are expected to vary in different HLA types and will need to be defined in order for sophisticated therapies to be possible.

Similarly to uveitis in the human, EAU in animals is genetically controlled (10). There are clear species-specific differences in sensitivity to uveitogenic proteins. For example, IRBP is a much more potent uveitogen than S-Ag for mice, while the reverse is true in guinea pigs, and both proteins are strongly uveitogenic in the Lewis rat (12–15). Within each species, strain dependence of susceptibility is apparent that is at least in part due to MHC control. Susceptible H2 haplotypes are $H2^b$, $H2^d$, $H2^k$, and $H2^r$. MHC control of susceptibility to EAU in the $H2^k$ haplotype was tentatively mapped to the IA subregion (a homologue of human HLA-DQ), with modifying effects from the IE locus (homologous to human HLA-DR) (15).

To better study the role of HLA molecules in the pathogenesis of uveitis, we undertook to develop a "humanized" model of EAU. To this end, we induced EAU in mice expressing transgenic (TG) HLA-DR3, -DR4, -DQ6, or -DQ8 molecules in the absence of the endogenous class II (*Aβ0*) (16). These mice positively select a repertoire of T cells expressing various Vβ T cell receptors that can identify immunogenic peptide epitopes similar or identical to human subjects of the same HLA-DR or -DQ genotype (16, 17). We show that all the tested HLA TG mice developed EAU after immunization with IRBP. In addition, HLA-DR3 TG mice developed severe uveitis after immunization with S-Ag, to which wild-type mice are highly resistant. Antibody blocking studies confirmed that the antigens were being recognized by the T cells in the context of the human HLA molecules. Furthermore, recognition of peptides derived from S-Ag and peptides derived from IRBP by HLA TG mice differs from recognition by wild-type mice and has some similarity to that of uveitis patients. Finally, as in classical EAU, the disease can be transferred by immune cells having a Th1 phenotype, but not by immune serum.

This new humanized model of uveitis offers a more relevant approximation of human uveitis than hitherto available rodent models and will facilitate the characterization of uveitogenic epitopes presented by different HLA class II types.

Methods

Animals. HLA-DR4, -DQ6, and -DQ8 single TG mice were developed at the Mayo Clinic and have been described previously (18–20). HLA-DR3 mice originally developed by Günter Hammerling and associates (21) were bred onto the Aβ0 background at the Mayo Clinic. The HLA-DR3 TG mice that carry the *HLA-DRA*0103* and *DRB1*0301* genes in an MHC class II–negative H2-Aβ0 background (*Aβ0.DR3*) express the human DR3 antigen as their only MHC class II molecule (22). HLA-DR4 TG mice had the HLA-DRB1*0401 gene in an Aβ0 background, modified to express IE molecules by insertion of the gene for the IE α chain from the $H2^k$ haplotype ($E\alpha^k$/$E\beta^b$.*Aβ0.DR4*) (18). Thus, they coexpressed murine H2-IE and human HLA-DR4 molecules. HLA-DQ6 TG mice carried the *DQA1*0103* and *DQB1*0601* transgenes in an Aβ0 background (Aβ0.DQ6) (19). Similarly, HLA-DQ8 TG mice had the *DQA1*0301* and *DQB1*0302* human genes (*Aβ0.DQ8*) (20, 23). Both HLA-DQ TG mice expressed the human DQ molecule as their only MHC class II antigen (Table 1). Controls were DR3- and DR4-negative littermates, Aβ0 mice, Aβ0 mice expressing IE antigens ($E\alpha^k$/$E\beta^b$.Aβ0), and C57BL/10 or C57BL/6 mice. DR3-negative littermates did not express any MHC class II molecules, their genotype being equivalent to that of Aβ0 mice. DR4-negative littermates expressed the IE molecule, having a genotype comparable to $E\alpha^k$/$E\beta^b$.Aβ0. These mice had the H2-IE molecule as their only MHC class II antigen. H2-IA^b is the only MHC class II molecule expressed by the parental strain C57BL/10, which has a defective gene encoding the Eβ chain and fails to express IE molecules (Table 1).

Animals were bred and maintained at the NIH or at the Mayo Clinic under specific pathogen–free conditions and were given water and chow ad libitum. The care and use of the animals was in compliance with institutional guidelines.

Genotyping. Mice were screened for presence of the human HLA molecule by PCR as described (24, 25). Expression of the human or murine MHC antigens was quantified by flow cytometric analysis of lymph node cells and splenocytes using FITC-conjugated anti-DR (clone L243), anti-DQ (clones Tü 39 and Tü 169), anti-IA (clone AF6-120.1), or anti-IE (clone 14-4-4S) antibodies (BD Pharmingen, Franklin Lakes, New Jersey, USA) as recommended by the manufacturer. Mice that did not demonstrate expression of HLA molecules by flow cytometry were excluded from analysis.

Antigens and reagents. Bovine IRBP (bIRBP) was purified from retinal extracts as described (26) by affinity

chromatography on ConA followed by ion exchange chromatography on a Pharmacia MonoQ column. Bovine S-Ag (bS-Ag) was prepared from the ConA column flowthrough as follows. The extract was dialyzed against 10 volumes of 10 mM HEPES, 15 mM NaCl, 1 mM EDTA, 1 mM benzamidine, pH 7.0, with the buffer changed once. S-Ag was purified by the method of Buczylko and Palczewski (27) with modifications described by Puig et al. (28). The final elution from the heparin-agarose column was via a gradient from 10 mM HEPES and 15 mM NaCl, pH 7.0, to 10 mM HEPES and 400 mM NaCl, pH 7.0. Purified bS-Ag was pooled based on an OD of 278 nm. Preparation of the recombinant human S-Ag (hS-Ag) was described previously (29). Pertussis toxin and CFA were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). *Mycobacterium tuberculosis* strain H37RA was purchased from Difco Laboratories (Detroit, Michigan, USA). Twenty-residue peptides overlapping by ten residues and spanning the first homologous repeat of human IRBP (hIRBP) (35 kDa) (30) and the entire hS-Ag (48 kDa; AnaSpec Inc., San Jose, California, USA) were synthesized by conventional solid-phase techniques as described (30, 31).

EAU induction and scoring. EAU induction and scoring was performed two ways: by active immunization and by adoptive transfer. For active immunization, mice were injected subcutaneously with 200 μg of bIRBP, bS-Ag, or recombinant hS-Ag emulsified (1:1, vol/vol) with CFA (Sigma-Aldrich) that had been supplemented with *M. tuberculosis* strain H37RA (Difco Laboratories) to a final concentration of 2.5 mg/ml. Concurrent with immunization, 0.2 μg of pertussis toxin was injected intraperitoneally. Eyes from IRBP-immunized mice were collected 21–28 days after immunization, and eyes from S-Ag–immunized animals were collected after 28–35 days. Eyes were fixed for 1 hour in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde. Fixed and dehydrated tissue was embedded in methacrylate. Sections (4–6 μm) were cut through the pupillary-optic nerve plane and then stained with standard H&E.

Quantitation of disease was performed in a masked fashion using criteria described previously (32). Briefly, eyes were assigned a score ranging from 0 to 4 depending on the extent of inflammation and tissue damage. The minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroid, vitreous, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue, such as vasculitis, granuloma formation, retinal folding and/or detachment and photoreceptor damage. The maximal grade of 4 reflects extensive retinal damage with complete destruction of the photoreceptor cell layer.

For induction of EAU by adoptive transfer, HLA-DQ8 and HLA-DQ6 mice were immunized with IRBP and HLA-DR3 mice were immunized with S-Ag using the uveitogenic protocol described above. On day 14 after immunization, immune serum was collected, titrated for antibody content by ELISA, and stored at 4°C until infusion into naive recipients. Lymph node and spleen cells were pooled within a group and were cultured for 3 days as described previously (33) with an optimal concentration of the immunizing antigen (30 μg/ml IRBP or 10 μg/ml S-Ag) and 5 ng/ml recombinant IL-12. Naive recipient mice of the appropriate genotype were infused intraperitoneally with 60×10^6 to 100×10^6 cultured cells. One milliliter of immune serum was injected intravenously as two doses of 0.5 ml administered concurrently with the cells and 48 hours later. Disease development was evaluated by fundoscopy and scored on a scale of 0–4 as described previously (32). EAU was confirmed by histopathology in eyes harvested after 16–18 days.

Delayed-type hypersensitivity responses. Two days before the termination of an experiment, mice received 10 μg of the appropriate antigen in a volume of 10 μl intradermally into the pinna of one ear. The other ear was injected with PBS. Ear swelling was measured 48 hours later with a spring-loaded micrometer.

Table 1
MHC class II genotype and phenotype of mouse strains used in this study

Strain	HLA transgene[A]	MHC class II phenotype	MHC class II restriction element	Reference
Test strains				
DR3	*DRA*0103; DRB1*0301*	Aβ0.DR3$^+$	HLA-DR3	(21)
DR4	*DRB1*0401*	Eα^k/Eβ^b.Aβ0.DR4$^+$	HLA-DR4/H2-IE$^{k/b}$	(18)
DQ6	*DQA1*0103; DQB1*0601*	Aβ0.DQ6$^+$	HLA-DQ6	(19)
DQ8	*DQA1*0301; DQB1*0302*	Aβ0.DQ8$^+$	HLA-DQ8	(20, 23)
Control strains				
DR3$^-$ littermates	-	Aβ0.DR3$^-$	-	
DR4$^-$ littermates	-	Eα^k/Eβ^b.Aβ0.DR4$^-$	H2-IE$^{k/b}$	
Aβ0	-	Aβ0	-	(51)
Eα^k/Eβ^b.Aβ0	-	Eα^k/Eβ^b.Aβ0	H2-IE$^{k/b}$	(52)
C57BL/10 or C57BL/6	-	H2-IA^b	H2-IA^b	

[A]The TG strains are on the C57BL/10 background.

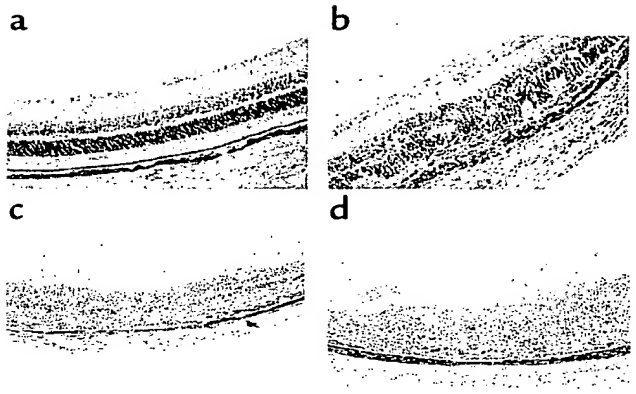

Figure 1
Histopathological features of EAU in HLA TG mice. (**a**) Normal eye. The retinal layers are ordered and well preserved. (**b**) Disease score 2 in C57BL/10 mice immunized with IRBP. The retinal architecture is disorganized, inflammatory cell infiltration is present, and the photoreceptor layer is damaged. (**c**) Disease score 3 in DR4 TG mice immunized with IRBP. Note extensive destruction of the photoreceptor cell layer. (**d**) Very severe disease (score 4) in DR3 TG mice immunized with S-Ag. Note complete destruction of the photoreceptor cell layers including photoreceptor cells (H&E, ×200).

Lymphocyte proliferation assay and antibody blocking. Lymph nodes draining the immunization site (inguinal and iliac) were collected at the termination of each experiment and were pooled within each group. Triplicate 0.2-ml cultures containing 5×10^5 cells/well were stimulated with 30 μg of IRBP or 20 μg of S-Ag in 96-well round-bottomed plates in RPMI 1640 (BioWhittaker Inc., Walkersville, Maryland, USA) supplemented as described and containing 1% mouse serum (32). For proliferation to bIRBP, 20 mg/ml α-methyl-mannopyranoside (Sigma-Aldrich) was included to neutralize any possible traces of Con A that might leach from the column used in the initial stages of IRBP purification. This concentration of α-methyl-mannopyranoside had no adverse effect on cell proliferation. Results are presented as stimulation indexes, calculated as average cpm of triplicate cultures with antigen, divided by the average cpm of triplicate cultures with medium, ± SE.

The restriction element involved in antigen presentation was assessed by antibody blocking assays using purified antibodies to DR (clone L243), DQ (clone Tü 39 or Tü 169), IA (clone AF6-120.1), or IE (clone 14-4-4S) (BD Pharmingen). The antibodies were added at a concentration of 10 μg/ml to wells containing the cells in the presence or absence of the appropriate stimulating antigen. The cultures were incubated for 60 hours and were pulsed with ^{3}H thymidine (1.0 μCi/10 μl/well) for the last 18 hours.

T cell epitope mapping. Twenty-residue peptides overlapping by 10 residues and representing the linear sequence of the first repeat of hIRBP or the entire sequence of the hS-Ag molecule were used (30) (AnaSpec Inc.). HLA-DQ8 TG, HLA-DR3 TG, or wild-type C57BL/6 mice ($H2^b$) were immunized with a uveitogenic regimen of IRBP or S-Ag, respectively. Spleens were harvested on day 10 after immunization and were pooled from several mice within each group. Cells were tested in a standard proliferation assay, as described above, against a 10-μM concentration of peptides derived from the immunizing antigen. Specific counts (Δ cpm) were calculated after subtraction of background cpm.

Determination of lymphokine content in culture supernatants and antibodies in immune serum by ELISA. Cytokines were determined in supernatants of lymph node cells collected on day 14 after immunization and cultured with an optimal concentration of the immunizing antigen as for the proliferation assays above. Supernatants were collected after 48 hours. The presence of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, and TNF-α in the supernatant of the stimulated cells was measured by multiplex ELISA using the Pierce SearchLight technology (Pierce Boston Technology, Woburn, Massachusetts, USA) (34) (http://www.searchlightonline.com). Serum antibodies were assayed on IRBP- or S-Ag–coated plates (5 μg/100 μl/well) using HRP-labeled goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, California, USA) as developing antibody and 3,3′5,5′-tetramethylbenzidine substrate (100 μl/well; Endogen Inc., Woburn, Massachusetts, USA).

Statistical analysis. Statistical significance of differences in disease scores were calculated using Snedecor and Cochran's test (35) for linear trend in proportions, with each mouse (average of both eyes) as one statistical event. This is a nonparametric test that generates its *P* values by frequency analysis of the number of individuals at each possible score, thus taking into account both severity and incidence of disease. Delayed hypersensitivity and lymphocyte

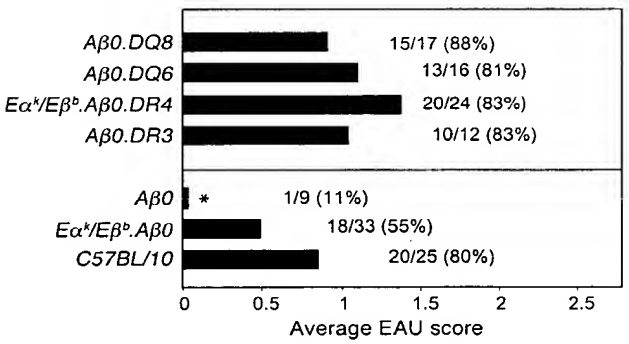

Figure 2
Susceptibility of HLA TG mice to IRBP-induced EAU. Bars are average of disease scores. Disease incidence (positive among total mice) is next to each bar. The HLA TG strains are in the top panel and the control strains are in the bottom panel. If only one eye showed disease the animal was scored as positive and its score was recorded as the average of both eyes. Shown are combined results of five experiments. Significant difference ($P \leq 0.05$) from C57BL control is indicated by an asterisk.

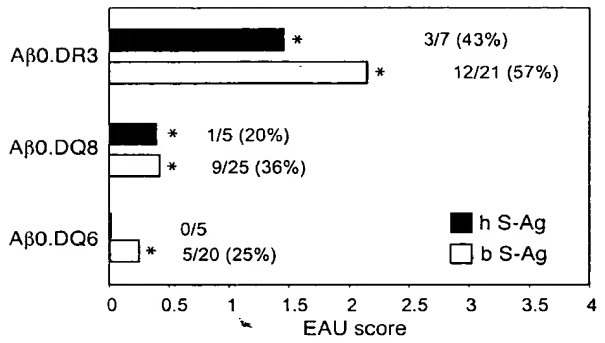

Figure 3
Susceptibility of HLA TG mice to S-Ag–induced EAU. Shown are combined results of five experiments using bS-Ag and one experiment using recombinant hS-Ag. Other TG strains were negative. Control *Aβ0* mice immunized with bS-Ag (n = 6) and wild-type C57BL/10 mice immunized with either hS-Ag (n = 4) or bS-Ag (n = 19) failed to develop disease. Significant differences ($P \leq 0.05$) from C57BL and Aβ0 controls are indicated by asterisks.

proliferation data were analyzed using an independent t test. Probability values of $P \leq 0.05$ were considered to be significant.

Results

HLA TG mice are susceptible to IRBP-induced uveitis. HLA TG mice were immunized with a uveitogenic protocol of IRBP and eyes were harvested for histopathology 21–28 days after immunization. Typical EAU was induced in all HLA TG strains. Histopathology (Figure 1, b and c) was grossly similar to that observed in wild-type mice on the C57BL/10 background, showing mononuclear and polymorphonuclear cell infiltration, vitritis, choroiditis, and varying degrees of photoreceptor cell damage. Disease severity in individual mice varied from 0.5 to 3, and incidence was about 80% (Figure 2). These results were not different from incidence and severity scores observed in the control strains. *Aβ0* mice, which do not express any MHC class II molecules but do express murine class I molecules, had little to no disease, with only one mouse of 11 showing trace infiltration in one eye, most likely representing a mild spontaneous inflammation unrelated to the experimental manipulations. We do not favor the possibility that this represents presentation of IRBP through the class I pathway because these mice had negligible IRBP-specific delayed-type hypersensitivity and proliferative responses (data not shown), suggesting that in the absence of class II, IRBP immunization fails to elicit a significant response.

Since no apparent differences in disease parameters were observed in HLA TG mice expressing or not expressing the murine IE molecules, their role when a human class II molecule is present appears to be subordinate. Nevertheless, when a human class II is absent, such as in the *Eα^k/Eβ^b.Aβ0* mice, the presence of murine IE alone is sufficient to permit EAU development.

DR3 TG mice are susceptible to S-Ag–induced EAU. Uveitis patients frequently exhibit strong lymphocyte responses to S-Ag but only sporadic responses to IRBP (12, 36). Although S-Ag is uveitogenic in other animal species, numerous strains of mice tested thus far proved to be highly refractory to disease induction with S-Ag (14, 15). Interestingly, HLA-DR3 TG mice immunized with bS-Ag developed very severe disease, with many individual mice reaching disease scores of 4, corresponding to complete destruction of the photoreceptor cell layer and extensive damage to the other retinal layers (Figure 1d and Figure 3). HLA-DQ6 and -DQ8 mice developed some disease as well, albeit with much lower scores and lower incidence (Figure 3). None of the other TG strains or any of the six control *Aβ0* mice developed disease after immunization with S-Ag (data not shown). Wild-type mice were also refractory to disease, confirming previously published data (14) (not shown). Onset of S-Ag–induced disease in the DR3 TG mice, which characteristically developed high disease scores, appeared to be delayed by several days to a week in comparison to the IRBP-induced disease. This is similar to the earlier onset of IRBP-induced EAU than of S-Ag EAU as observed in rats, and may reflect a difference in accessibility of the target antigen (S-Ag is intracellular in the photoreceptor cells, whereas IRBP is secreted into the interphotoreceptor matrix) (37).

There is about 80% homology in the amino acid sequence between human and bS-Ag. To test whether this translated to differences in pathogenicity, we immunized HLA TG mice with recombinant hS-Ag.

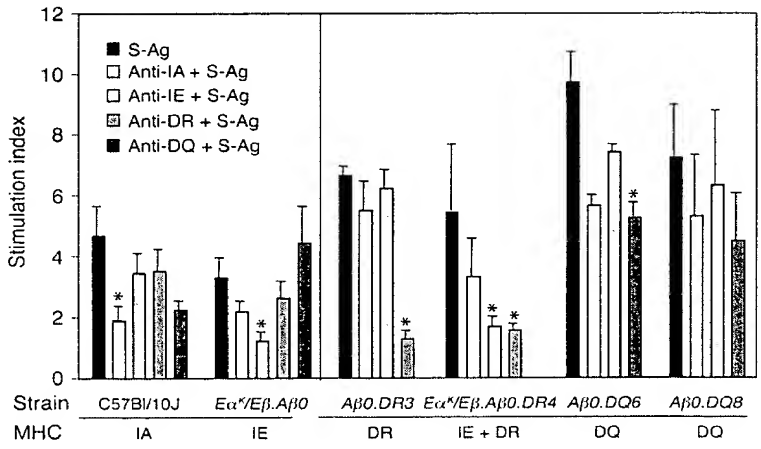

Figure 4
Proliferative response of lymph node cells to bS-Ag. Average of stimulation indices observed in several experiments is shown. Error bars indicate SE. Significant difference from control ($P \leq 0.05$) is indicated by an asterisk. Background counts varied between 1,500 and 3,000 cpm depending on the group.

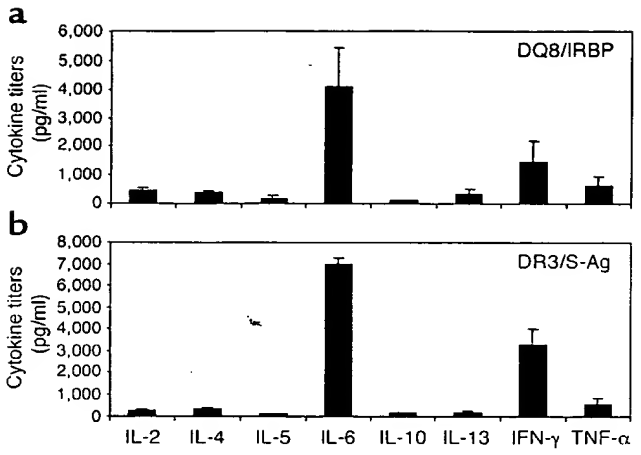

Figure 5
Cytokine responses. Cytokine production by lymph node cells from HLA-DQ8 TG mice stimulated with bIRBP (**a**), and lymph node cells from HLA-DR3 TG mice stimulated with bS-Ag (**b**), measured by multiplexed ELISA. Shown are titers in pg/ml averaged from two experiments.

DR3 and DQ8 TG mice developed EAU after immunization with the recombinant hS-Ag with scores similar to the EAU developed in response to bS-Ag (Figure 3). This finding is of particular importance in view of the strong responses of human patients to S-Ag, that had presumably developed as responses to the autologous human molecule. HLA-DR4 TG mice and all the control strains, including the wild type, were resistant (data not shown).

The retinal antigen is presented and recognized on human MHC molecules. Because DR (though not DQ) strains retain the mouse Eβ, and in the case of DR4 also the Eα molecule, it was necessary to verify that antigen can be presented productively on the human class II molecules. This was done by lymphocyte proliferation blocking studies using appropriate monoclonal antibodies. Draining lymph node cells from mice immunized with IRBP or bS-Ag were collected and were stimulated in culture with the corresponding antigen. Proliferative responses were observed in all strains except Aβ0, which is devoid of any class II molecules. In HLA-DR3, -DQ6, and -DQ8 TG mice, proliferation in response to S-Ag was blocked by specific monoclonal antibodies to human, but not to mouse, MHC class II molecules (Figure 4). In $E\alpha^k/E\beta^b$.Aβ0.DR4 TG mice, which coexpress DR and IE molecules, both anti-DR and anti-IE antibodies abrogated proliferation, indicating that both class II molecules were functionally involved in antigen presentation. A similar pattern was observed with HLA TG lymph node cells of mice immunized with IRBP (data not shown). A cytotoxic effect of antibodies was excluded because the anti-HLA antibodies did not interfere with proliferation of wild-type lymphocytes to antigen and because background counts in the presence of antibodies without antigen did not differ from background in presence of medium alone (data not shown).

It should be pointed out that strong proliferative responses were seen in all genotypes except Aβ0, irrespective of whether or not they developed disease. Similarly, all strains except Aβ0 mounted delayed-type hypersensitivity responses against S-Ag and IRBP (data not shown). This indicates that not only pathogenic but also nonpathogenic epitopes of the retinal antigens in question were functionally presented by human class II molecules.

Pathogenesis of EAU in HLA TG mice: a cell-mediated response with a Th1-predominant cytokine profile and a modifying role for antibodies. Studies of EAU in wild-type mice indicated a central role for a Th1-type response in pathogenesis of disease (1). To evaluate the responses of HLA TG mice to uveitogenic immunization, we measured the cytokines in supernatants of lymph node and spleen cells from immunized mice that were stimulated in vitro with the immunizing antigen. We selected as representative strains the DQ8 TG mice

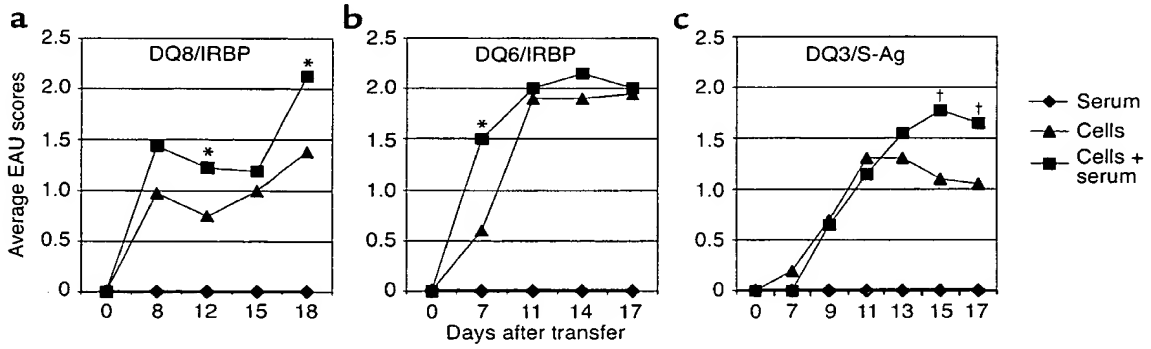

Figure 6
Adoptive transfer of cells and/or serum. Recipient mice were infused with serum, cells, or cells and serum from immunized syngeneic donors. EAU development was followed by fundus examination. (**a** and **b**) Disease scores of HLA-DQ8 and HLA-DQ6 recipients whose donors were immunized with bIRBP. (**c**) Disease scores of HLA-DR3 recipients whose donors were immunized with bS-Ag. Shown is one of two representative experiments with five mice per group. *Statistically significant difference in scores from cells alone ($P \leq 0.05$). †Trend ($P \leq 0.1$).

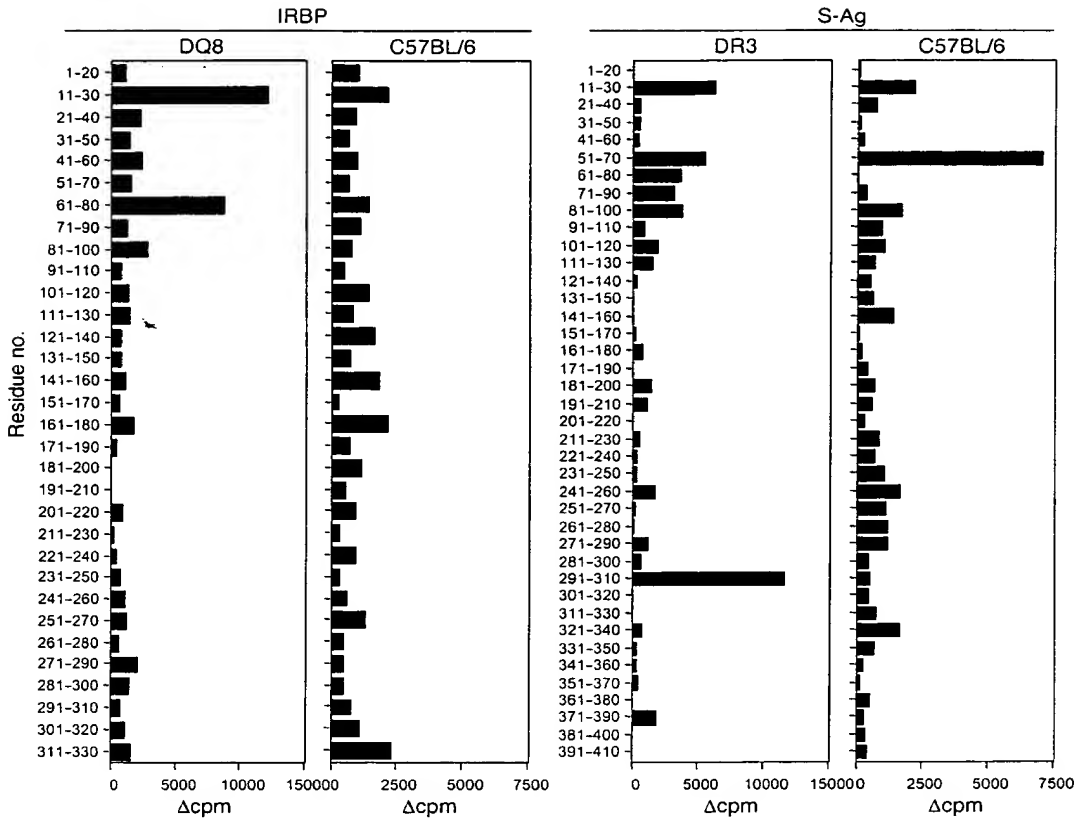

Figure 7

Mapping of T cell epitopes. Epitope recognition by DQ8 mice immunized with bIRBP and DR3 TG mice immunized with bS-Ag was compared with that of C57BL/6 (IA^b) wild-type controls. Antigen-specific responses were recalled with overlapping peptides representing the first repeat of human IRBP or the whole hS-Ag. Shown are specific counts as averaged from two to three repeat experiments for each strain of mice. Background counts varied from 450 to 3,000 cpm depending on the group.

immunized with bIRBP and the DR3 TG mice immunized with bS-Ag, which were high responders for the respective antigens. Both strains produced very high levels of IL-6, which was also typical of wild-type C57BL/6 mice (not shown). Of the cytokines typifying a Th1 or a Th2 response, IFN-γ and TNF-α were predominant over IL-4, IL-5, and IL-13. DR3 mice produced relatively more IFN-γ than did DQ8 mice (Figure 5, a and b). Thus, EAU induced in HLA TG mice appears to be associated with a Th1-predominant cytokine response profile, analogous to the "classical" model of EAU in wild-type mice.

In wild-type mice, EAU can be adoptively transferred by immune cells but not by immune serum. To test whether the pathogenesis of EAU in HLA TG mice was cell- or antibody-mediated, we adoptively transferred cells, serum, or cells and serum together from immunized to naive syngeneic mice which were then followed by fundus examination. Activated lymphocytes from immunized mice cultured with antigen in the presence of IL-12 (33) were able to transfer EAU to naive syngeneic recipients (Figure 6). None of the mice that received high-titer immune serum alone (antibody titers between 1×10^{-6} and 1×10^{-7}, see Methods) developed disease. Interestingly, when cells and serum were combined, the serum was able to slightly but consistently raise disease scores compared with cells alone, suggesting that antibodies can modify the course of disease.

T cell epitope recognition of IRBP and S-Ag epitopes in HLA TG mice. The results described above indicated that T cells responding to retinal antigen presented on HLA molecules were involved in the disease process in HLA TG mice. Because the class II–restricted T cell repertoire is selected in these mice by human class II, we decided to examine epitope recognition of HLA TG mice. We chose as representative strains those with high disease susceptibility for each antigen: DQ8 for IRBP and DR3 for S-Ag. Mice were immunized with native bovine antigen, and spleen cell responses were recalled in vitro with a panel of overlapping peptides representing the entire first repeat of hIRBP or the entire sequence of hS-Ag. The responses were compared with those of $H2^b$ haplotype wild-type mice (C57BL/6). Our decision to immunize with bovine proteins and recall with human peptides (although human recombinant proteins are available) was based on the reasoning that

in a human-human or a bovine-bovine combination, the strongest responses would represent recognition of nonconserved epitopes foreign to the mouse. In the bovine-human combination, the responses would be more likely to represent recognition of conserved epitopes shared with the autologous antigen.

The results showed distinct differences between epitope recognition of HLA TG and wild-type mice, suggesting that a different T cell repertoire had been selected (Figure 7). IRBP peptides 11-30 and 61-80 were immunodominant in DQ8TG mice but not in wild-type mice. S-Ag peptide 291-310 (NRERRG-IALDGKIKHEDTNL) appeared to be immunodominant in DR3TG mice. This peptide was weakly recognized by wild-type mice, which instead responded strongly to peptide 51-70. It is of interest to note that peptide 291-310 of S-Ag partly overlaps with the previously characterized S-Ag peptides M 303-320 and N 281-302, which elicit responses in lymphocytes of patients affected by different uveitic diseases (11, 31).

Discussion

The present study describes a new, "humanized" model of EAU developed in HLA TG mice in which disease-relevant epitopes appear to be largely restricted by the human class II molecules. Evidence that this is indeed the case is provided by (a) lack of class II molecules other than the human one in the HLA-DQ6, HLA-DQ8, and HLA-DR3 mice; (b) lack of disease in the control Aβ0 mice lacking the human class II; (c) the ability of the appropriate anti-HLA antibodies to block lymphocyte proliferation of HLA TG mice in response to the immunizing retinal antigen; and (d) distinct differences in epitope recognition between HLA TG and wild-type mice, suggesting differences in the selected Ag-specific repertoire.

Ours is not the first attempt at establishing a humanized model of uveitis. Uveitis is a heterogeneous group of diseases affecting different parts of the eye and showing association with various HLA class I or class II molecules (38). HLA-B27 is strongly associated with anterior uveitis that accompanies ankylosing spondylitis. HLA-A29 confers an increased risk of birdshot chorioretinopathy that is in some cases up to 224 times higher than the general population (39). Sympathetic ophthalmia is associated with HLA-DR4, and intermediate uveitis (unrelated to multiple sclerosis) with HLA-DR3 (3–8, 39, 40). The relevant human HLA molecule does not necessarily precipitate the appearance of symptoms in the animal carrying it as a transgene. Although HLA-B27 TG rats and mice do develop spontaneous spondyloarthropathies, they do not develop either spontaneous or bacterially induced anterior uveitis (41–43). In contrast, the recently described HLA-A29 TG mice develop spontaneous uveitis with striking histological similarity to the HLA-A29–associated disease in humans. This supports the conclusion that the HLA-A29 molecule itself participates in the pathogenesis of birdshot chorioretinopathy (41). The EAU model in HLA class II TG mice described here provides evidence in favor of an involvement of class II–restricted responses in human uveitis. No less importantly, this new model suggests that the same retinal antigens that are uveitogenic in animals may also be causally involved in human uveitic diseases and validates antigen-specific immunotherapies based on these antigens, such as the recent oral tolerance trial in which uveitis patients were fed S-Ag and in fact appeared to receive a clinical benefit (44).

Arguably the most interesting observation in the present study is the susceptibility of HLA-DR3 TG mice to disease induced with S-Ag. Of particular note is the finding that the T cell epitope recognition in these mice takes on a similarity to that of uveitis patients. DR3 TG mice but not wild-type mice responded to S-Ag peptide 291–310. This sequence partly overlaps with peptide M, a promiscuous S-Ag epitope spanning residues 303–320 that is uveitogenic in rats, guinea pigs, and primates, and is recognized by lymphocytes from uveitis patients but not by healthy controls (11, 12, 31, 45). Although human patients appear to exhibit much more frequent cellular responses to S-Ag than to IRBP, wild-type mice develop EAU with IRBP but are highly resistant to EAU induced with S-Ag. It has been proposed that the reason for this is central tolerance to S-Ag, owing to its abundance in the murine thymus (46), such that T cells capable of recognizing S-Ag are efficiently deleted from the repertoire. The dramatic change in susceptibility of HLA-DR3 TG mice to S-Ag, apparently stemming solely from substitution of the restricting class II molecule from mouse to human, suggests that other mechanisms may be at play. Thus, in addition to and independently of its potential to bring better understanding of the involvement of S-Ag in human uveitic disease, the uveitis model in HLA-DR3 mice also offers the opportunity to approach basic questions in the development of self-tolerance to retinal antigens. These questions will be the subject of a separate study.

The $E\alpha^k/E\beta^b.A\beta0$ mice (which served here as controls for the $E\alpha^k/E\beta^b.A\beta0.DR4$ TG mice) provide some new information about the role of IE molecules in the EAU model. It is apparent that IA molecules are sufficient to permit induction of EAU in the $H2^b$ haplotype, as mice on the C57BL background, which do not express IE molecules, develop disease. Our previous data in intra-H2 congenics tentatively mapped control of EAU to IA^k, with only modifying influence from IE^k (15). The present data show for the first time that the IE molecule by itself, without the presence of IA, is sufficient to confer the ability to develop EAU.

The interaction between the T cell coreceptor CD4 and MHC class II molecules plays a crucial role in intrathymic selection as well as peripheral activation of $CD4^+$ T cells. In our humanized EAU model,

murine CD4 molecules bind to the β2 domain of human MHC class II. It has been argued that species-specific amino acid differences might alter the interaction between mouse CD4 and human class II, resulting in reduced or modified responses (18, 47). Since we do not have human CD4 TG mice at our disposal, we attempted to partly address the effect of a homologous versus heterologous CD4–class II interaction by using DR4 chimeric mice. These mice express a hybrid DR4 molecule formed from the invariant portion of the murine IE molecule that contains the CD4 binding site, fused with the variable region that contains the antigen-binding pocket of the human HLA-DR4 molecule. Unlike $E\alpha^k/E\beta^b$.Aβ0.DR4 mice, these DR4 chimeric mice have no murine class II molecules and thus have only a homologous CD4–class II interaction. DR4 chimeric mice developed IRBP-induced EAU with an incidence and average score similar to that of nonchimeric DR4 TG mice and were resistant to S-Ag (data not shown). Thus, presence of a heterologous CD4–class II interaction in our model appears not to cause an altered pattern of susceptibility to disease.

The pathogenesis of EAU in the HLA TG mice appears to be similar to that of EAU in wild-type mice and reinforces the notion that cell-mediated responses may also be causally involved in human uveitis. In wild-type mice, uveitogenicity is associated with the Th1 response (1). Both IRBP-induced EAU in DQ8 TG mice and S-Ag–induced EAU in DR3 TG mice were associated with an IFN-γ–dominant response to their respective uveitogen. Interestingly, IL-2– and IFN-γ–producing $CD4^+$ and $CD8^+$ cells are detected in peripheral blood from patients with Behçet disease (48, 49). Disease could be transferred with Th1-polarized cells from primed individuals into naive recipients. Immune serum alone did not transfer disease, but in conjunction with the cells, injection of serum was able to slightly, but consistently, raise disease scores. This suggests that although the antibodies by themselves are unable to enter the eye through an intact blood-retinal barrier, once the blood-retinal barrier is breached by activated T cells, antibodies serve to modify the course of disease.

In summary, this is the first report of a humanized class II–restricted model of uveitis that includes a new mouse model for S-Ag–induced uveitis. This model validates the class II involvement in human uveitis and supports an etiological role for retinal antigens, which are uveitogenic in animals, in human disease. Identification of T cell determinants restricted to MHC molecules known to predispose to uveitis will permit better understanding of disease mechanisms and promises to facilitate development of antigen-specific therapies tailored to particular HLA haplotypes. Importantly, once autoantigenic epitopes have been identified, the disease model generated in these mice will permit manipulations to mechanistically elucidate the development of autoimmunity that are not possible in human patients (50).

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HLA-DR, DQ and T cell antigen receptor constant beta genes in Japanese patients with ulcerative colitis.

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We studied the T cell antigen receptor (TcR) constant beta chain genes on HLA typed Japanese patients with ulcerative colitis (UC). A TcR constant beta EcoRI 6.0-kb fragment was present in all Japanese UC patients (n = 17) but completely absent in the controls (n = 35) (chi2 = 47.6, P less than 0.001). The frequency of HLA-DR2 antigen was significantly higher in UC patients (85% versus 28% in controls, P less than 0.001). Furthermore, HLA-DQw1 antigen was also increased in UC patients (96% versus 60% in controls, P les than 0.001). However, HLA-DR4 antigen was significantly decreased in UC patients (12% versus 37%, P = 0.02). HLA-DR1 antigen was not found in UC patients and was present in only 15% of the controls. These results suggest that TcR beta chain and HLA-DQw1 antigen may be important in the pathogenesis of Japanese UC.

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Exhibit 7

Autoimmunity

Normally, individuals do not form potentially destructive Abs to their own cells, but only to foreign Ags. This is because the body has developed a tolerance to the Ags (other than Igs) normally present within self. This state of the immune tolerance to self Ags is maintained by a complex network of T and B lymphocytes and their regulatory products. However, in certain diseases, we can produce Abs to our own cell or tissue components. This type of Ab is called an **auto- Ab** and the diseases associated with Auto-Abs are called **autoimmune diseases**.

It is essential that the body be able to recognize self from nonself for survival. For example, the immune system can distinguish self MHC Ags on its own cells from those on foreign cells. Yet some physiological autoimmune reactivity against self-Ags (Abs) can occur and is normal. The auto-Abs produced by this action serve as physiological regulators of the immune system. Thus, auto-Abs can exist at normal physiological levels as components of the body's homeostatic mechanisms. It has been postulated that auto-Abs might also act as "biological taxis," transporting cellular breakdown products for their ultimate disposal. For example, myocardial infarcation (e.g., heart attack), we find apparently harmless auto-Abs to heart tissue, whose function is to clear away damaged heart tissue. But the appearance of normal auto-Abs is a carefully controlled event: if f goes awry and lymphocytes proliferate and large quantities of auto-Abs are produced , autoimmune disease can occur. The pathological breakdown of self tolerance will be discussed later.

THEORIES OF BREAKDOWN IN SELF-TOLERANCE

There are several ways that auto-Abs formation can be triggered. Scientists have been able to identify either the precipitating event that takes place just prior to the expression of disease or the underlying events that seen to eventually lead to a disease state, but not both . What was originally thought to be a simple matter of one common biological trigger for many autoimmune disorders is now known to be a puzzling assortment of triggers, which have to occur in the right space and time for disease to occur. For example, in juvenile diabetes, complement-fixing auto-Abs to the insulin producing islet cells of the pancreas cause their immune destruction with subsequent expression of the clinical symptoms of diabetes. When siblings of diabetics were tested, many possessed the same IgG auto-Abs directed against pancreatic B cells, but they showed no sign of disease. However, within 3 to seven years the siblings who tested positive for auto-Abs became frankly diabetic. This would indicate that there were other triggers that occur before the disease manifested itself; it was not just the presence of cytotoxic Abs that brought on the disease. But final sequence of events is still unknown.

Another example suggesting that the presence of auto-Ab alone is not sufficient to cause disease is autoimmune hemolytic anemia. In this disease, monoclonal auto-Abs are formed against normal red blood cell constituents. The Abs coat the erythrocytes, causing clumping, lysis and premature clearance by the spleen. The immunological test used to diagnose this condition is called **Coomb's test**, which can assess whether an individual's red blood cells are coated with IgG Ab. However, a positive Coomb's test occurs without any evidence of hemolytic disease In a small percentage of healthy blood donors. Thus, although it is necessary for red blood cells to be coated with auto-Ab in hemolytic anemia, other factors also must be involved in order for disease to manifest itself.

The triggers for autoimmune diseases are diverse and include immunological, genetic, viral, drug-induced and hormonal factors, acting singly or in combination, in time and space. At present many individual mechanisms have been identified, but how they interact with the immune network has not yet been elucidated. Mechanisms that have been shown to eventually cause a **breakdown of self tolerance include (1) infection of somatic tissue by viruses, (2) development of altered self-Ags due to**

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Exhibit 8

binding of certain drugs to cell surfaces (3) cross reactivity of some Abs to bacterial Ags and self-determinants, (4) development of newly exposed Ags in the body, (5) the influence of hormones, and (6) breakdown in the immune network that recognizes self.

A major focus of the immune system is to maintain self in the body. An event that could lead to a breakdown in self is **viral infection**. Since viruses can cause the display of viral Ags on the surface of body cells, viral antigenic expression could act to induce autoimmune diseases. Many animal viruses can a latent state, where they remain hidden and noninfective for long periods of time. However, in this state they might still influence the cell surface markers, which could lead to an autoimmune response. Viruses can also induce autoimmune diseases by polyclonal activation of lymphocytes, the release of subcellular organelles after viral lysis of the cell by antigenic mimicry or by functional impairment of regulatory immunocytes such as Ts or TH cells. Viral infections prior to disease have been associated with systemic lupus erythematosus (SLE), multiple sclerosis, and diabetes.

Another way to alter cell-surface antigenic determinants is by **binding of certain drugs**. Hemolytic anemia can be produced in susceptible people taking the antibiotic penicillin. Penicillin can bind to erythrocytes and Abs that develop to the drug can then bind to the foreign antigenic penicillin molecules. It has been suggested that the membrane-bound penicillin can initiate events that lead to the activation of lymphocytes, previously silence by immune suppresser activity. Subsequent Ab formation against erythrocyte Ags and complement activation can lead to hemolysis and the onset of anemia.

Autoimmune diseases can also be caused by the **formation of Abs that crossreact** with the host's Ags that crossreact with self-Ags in human somatic tissue. For example, in syphilis, caused by the spirochete *Treponema pallidum*, many people develop hemolytic anemia. It has been suggested that Abs raised against *Treponema* Ags can also crossreact with certain erythrocyte blood group Ags, thus bringing about the anemia. Antigenic mimicry is also seen in the pathology of Chaga's disease. There is evidence that Ags common to its causative agent, *Trypanosoma cruzi*, and human cardiac muscle produce the immunopathological lesions seen in this disease. In addition, crossreactions of Ags against *Streptococcus* Group A bacteria with human cardiac muscle are responsible for the myocardial effects of rheumatic fever.

Hormones seem to influence the expression of certain autoimmune diseases as well. It is known that hormones of the hypothalamus, thyroid and adrenal glands affect the homeostasis of the lymphoid system and responses to Ags, by as yet uncharacterized mechanisms.. SLE and RA (rheumatoid arthritis) preferentially afflict women, where as more men develop myasthenia gravis. The predisposing factors in these instances appear to be the sex hormones. It is known that testosterone is immunoenhancing; but how these hormones contribute to the disease state has not been elucidated.

Last, and perhaps most important, autoimmune diseases can be triggered by some **breakdown in the immune network** that strictly monitors the expression of self. The breakdown in the immune network can occur at many different levels. It could be at the level of expression of a defect in the functioning of the immunocyte.

At the level of **production defects**, there is also the possibility that germline or somatic mutations of the B or T lymphocytes, by adversely affecting the microenvironment of either the stem cells, some other precursor cell, or the differentiating lymphoid cell. It is known that thymic hormones, such as thymosin and thymopoietin, are essential for differentiation of T cells and their helper, inducer, suppressor, and cytotoxic subsets.

Defects in Ag recognition might also contribute to the expression of autoimmune diseases. For example, it has been suggested that the number of immunoreactive cells recognizing self-peptides and MHC molecules within the developing thymus might not be properly regulated. Consequently, cells (which should have been clonally deleted) might remain. These cells later could be stimulated inappropriately by combination with self-components altered by disease or other factors. Furthermore,

malfunction in the regulation of T cell expression could lead to contrasuppression of T cell function. It is believed that TS lymphocytes play an essential role in maintaining immune silence to certain self-components found in somatic tissue cells. Interference with (or contrasuppression of) TS cell activity could lead to autoimmune diseases by allowing the immune system to interact positively with self-Ags. Alternatively, enhanced TH cell activity could also disrupt the immune network. Unresponsiveness, to self-Ags could be maintained by self-tolerance at the level of the TH cell. If activated, these tolerant TH cells could be induced to activate B cells to produce auto-Abs.

The induction of autoimmunity could also be accomplished by bypassing T cells. For example, self reactive cells could be directly stimulated by **polyclonal activators** such as lipopolysaccharides (LPS), which can directly activate B lymphocytes. In this way, we could circumvent the tolerant T cells and by pass the regulatory mechanisms. This could lead to the direct activation of specific B cells, with su bsequent formation of Abs to self components. This has led to the suggestion that autoimmune diseases arise through a combination of polyclonal and Ag-specific stimulation. For example, polyclonal stimulation could cause expansion of B cell clones expressing surface receptors for self-Ags. Subsequent encounter with Ag would lead to activation . Moreover, it is possible that specific activation and self-Ag-driven selection of autoreactive B cells could occur.

Since proper Ag presentation is essential for Ab production by B cells and T cell expression of its subsets and effector molecules, it is possible that **macrophage detectors** could produce an autoimmune response. Defects in Ag presentation could tip the scales to effect a choice of TH over TS cells in response to a self-Ag. There is littler information of the functional state of macrophages in autoimmune disorders yet it is accepted that mononuclear phagocytes play an essential role in determining how Ag is processed and presented to lymphocytes which underlies immune competency.

Ag-processing macrophages also produce factors that influence the activities of lymphocytes. One of these is the monokine interleukin 1(IL-1), a mitogenic regulatory molecule that stimulates TH activity. If pathological signals cause an inappropriate release of lymphokines the immune system could become primed to react to self-Ags in a way that would lead to tissue destruction.

MECHANISM OF TISSUE DAMAGE IN AUTOIMMUNE DISEASES

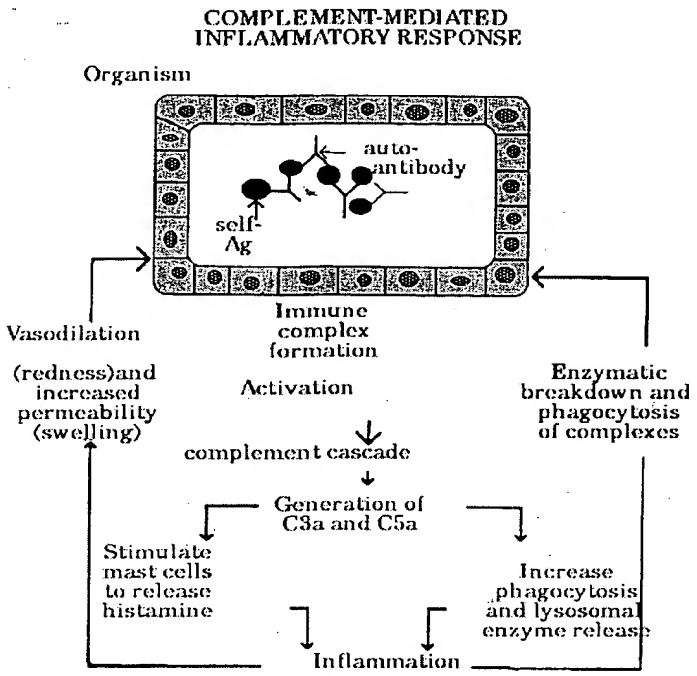
There are a variety of ways in which an autoimmune response can cause tissue damage. General mechanisms of action can be classified into the following groups: (1) damage by complement-fixing Abs raised against auto-Ags; (2) compromise of cellular function when auto-Abs bind to the cell surface receptors which mediate, degrade or block expression of differentiated function; (3) tissue damage when auto-Abs and soluble self-Ags form immune complexes and initiate a destructive inflammatory response; and (4) damage to cells through specific TC cell responses activated to destroy self-cellular Ags.

Auto-Abs directed against self-Ags located on the membrane of body cells might bind to these Ags, **activate serum complement**, and cause cytolysis. This occurs in autoimmune hemolytic anemia, when IgG auto-Abs bind to certain blood group Ags, fix complement, and destroy the erythrocytes, thereby causing anemia.

Auto-Abs can form against cell surface receptors, for example, hormone receptors. These auto-Abs can either mimic the function of the normal hormone, block hormone binding, or even degrade the receptor site. Thus, the formation of a receptor-auto-Ab complex alters the function and activity of the receptor. In myasthenia gravis, auto-Abs are produced to myocyte acetycholine receptors located at the neuromuscular junction. These Ags interfere with proper neurotransmission, diminish the contractility of the muscle cells and cause fatigue and weakness. In contrast, in thyrotoxicosis, binding of auto-Abs stimulates the thyroid gland, mimicking the action of the normal hormone.

Auto-Abs can be produced against soluble self-Ags, which can **form immune complexes**. This might

lead to activation of the complement cascade, with the formation of the anaphylactic and chemotactic fragments, C3a and C5a. Histamine is then released and phagocytic activity is increased, causing an inflammatory response that is destructive to tissues at the site of immune-complex formation. This is the usual series of events during acute episodes in SLE, when auto-Abs to DNA generate anti-DNA: DNA complexes with subsequent inflammation.



The recognition of self-Ags as immunostimulatory can lead to the **initiation and expansion of T lymphocyte subsets.** Populations of phagocytic cells ant TC cells would be drawn to the antigenic site and proceed to destroy cells displaying the specific self-Ags. An example of this type of immune mechanism is autoimmune thyroiditis, where accumulation of phagocytic and cytolytic cells can be found in the thyroid lesions.

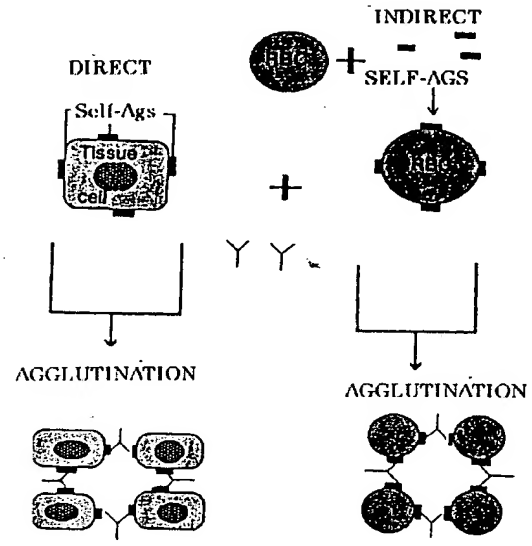
DIAGNOSTIC TESTING

Although, selective methods can be employed to detect each autoimmune disease, testing generally falls into three categories: (1) immunofluorescence, (2) agglutination, and (3) radioimmunoassay.

The indirect **immunofluorescence**(IF) test is usually used in the detection of autoimmune disorders. This test may be employed to determine the presence of auto-Abs in serum. Frozen tissue samples, displaying the suspected self-Ag, are incubated with a sample of the person's serum. If the serum contains auto-Ab, the Ig binds to the surface self-Ags. Then anti-human IgG Ab, tagged with a fluorescent dye, is added. If auto-Abs are bound to the tissue cells, the fluorescent anti-IgG molecule will bind to the Fc portion of these auto-Abs and the cells will fluoresce when viewed with a fluorescence microscope. Cells that are unaffected by auto-Ab will not fluoresce and will appear dark against the glowing cells that are affected by autoimmune disease. This type of testing is used to diagnose autoimmune diseases like Hashimoto's thyroiditis, juvenile diabetes, Goodpasture's syndrome, myasthenia gravis, RA and SLE.

In the direct **agglutination test**, serum is added to a suspension of cells that have the surface self-Ag to be tested.

INDIRECT AND DIRECT AGGLUTINATION

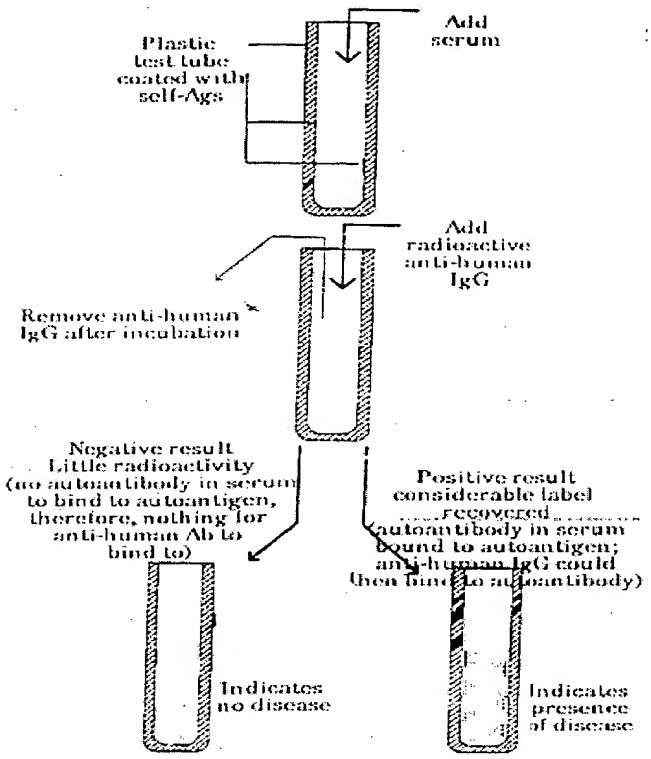


If the individual's serum contains the specific auto-Abs, Ig will bind and, at the appropriate Ab concentration, the cells will become cross linked. This will cause agglutination , and the cells will form a mat at the bottom of the test well. Auto-Abs attached to a patient's cells can be detected by the addition of a second Ab and observed for agglutination.

Selective soluble self-Ags can also be used to assay auto-Abs by attaching them to the surface of red blood cells. This latter type of agglutination test is called passive or indirect hemagglutination. Agglutination tests are commonly used to detect RFs (RA), thyroglobulin auto-Abs (thyroiditis), and red blood cell Abs (autoimmune hemolytic anemia).

The **radioimmunoassay** (RIA) is a very sensitive technique for detecting small quantities of auto-Abs in the serum of persons suspected of having autoimmune disease.

QUANTIFYING AUTOANTIBODY BY RIA



Ag can be adsorbed onto the inner surface of a plastic tube and test serum can then be added. If the serum contains auto-Abs specific for the bound Ag, it will bind to the Ag. A radioactively labeled secondary Ab (anti-human IgG) can then be added which attaches only to the Fc portion of IgG. The tube is washed and then the radioactivity in the tube is measured in a gamma counter. If the serum does not contain auto-Abs, then none of the radioactive IgG should bind, and there should be little radioactivity in the tube. However, small amounts of auto-Ab in the serum should lead to the binding of labeled anti-IgG, which can be detected in the gamma counter. RIAs are used to determine the presence of intrinsic factor (pernicious anemia), anti-DNA Abs (SLE), and antithyroglobulin IgG (thyroiditis). These tests can also be used to screen people who are considered at risk for a specific autoimmune disease.

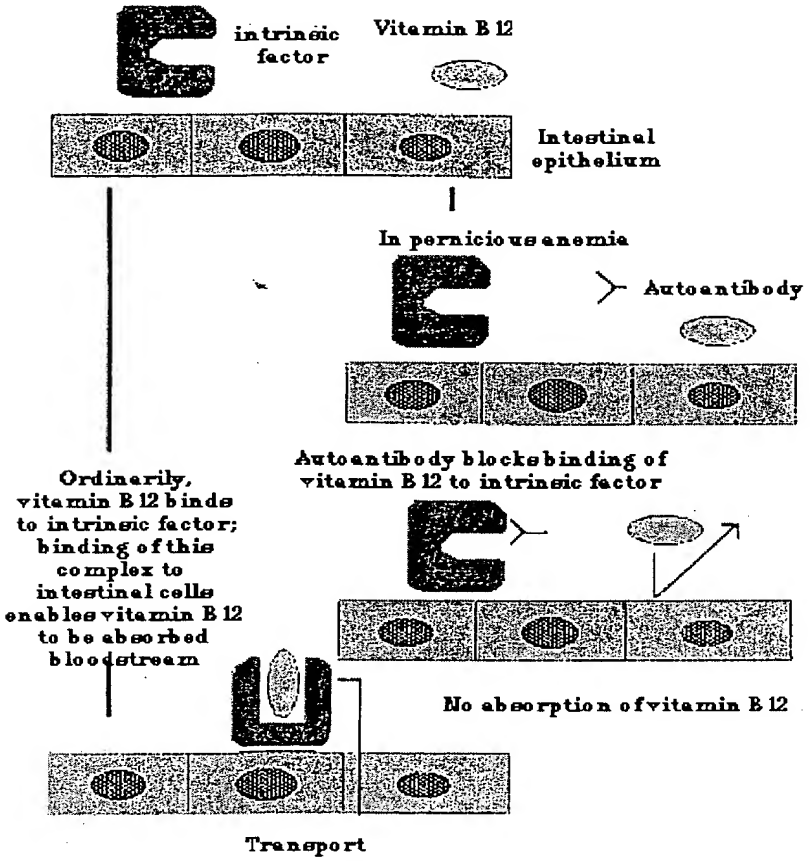
SELECTED AUTOIMMUNE DISEASES: ORGAN SPECIFIC

The tissue destruction that occurs is restricted to the specific Ags localized in that organ. Hence, the specific auto-Ab will not attack other organs. There can be overlap with other organ-specific diseases, which predispose the individual to other autoimmune diseases. In such cases the auto-Abs are separate and the organ destruction is distinct. An example of this situation occurs with the increased predisposition of individuals with pernicious anemia to developing thryroiditis. The auto-Abs and organs affected are different, but the expression of one disease seems to lead to further breakdown in self-tolerance in other organ systems.

Autoimmune Anemias

In **pernicious anemia** , an Ig G auto-Ab can be made that binds to a specific receptor secreted by stomach cells that is responsible for transporting vitamin B12 in the small intestine.

IMMUNOLOGICAL MECHANISM IN PERNICIOUS ANEMIA



The receptor is a membrane-bound protein called intrinsic factor. The binding of auto-Ab to intrinsic factor can block transport of vitamin B12. Vitamin B12 can not be synthesized by the body, but is supplied by diet and is transported across the intestinal wall principally by intrinsic factor. Vitamin B12 is essential for the proper maturation of erythrocytes. Depriving the body of vitamin B12 leads to the release of immature erythrocytes that do not function well as transporters of oxygen. If pernicious anemia is not treated with injection of vitamin B12 (delivered to the blood stream, thus by passing intestinal absorption), the individual will die.

Autoimmune hemolytic anemia encompass a wide spectrum of disease states that are typified by the production of auto-Abs that can bind to erythrocyte blood group Ags. Autoimmune anemias that are not drug induced fall into two major classes: warm and cold Ab types. Warm Abs are mainly IgG auto-Abs but can also include Ig M auto-Abs that will optimally agglutinate erythrocytes at body temperature . Cold Abs are IgM auto-Abs that agglutinate erythrocytes only when the blood is chilled.

Although the etiology is unknown, warm-reactive auto-Abs, are usually formed against the Rh system Ags. The binding of Ab to the erythrocytes in the body usually results in rapid clearance of sensitized erythrocytes in the spleen, thereby causing anemia. In contrast, cold-reactive auto-A bs, whose specificity is against the Ii blood Ag system, can bind, fix complement, and cause hemolysis in areas where peripheral circulation temperatures fall below 37oC .

Agglutination can also occur. Consequently, in the extremities (hands, feet, and ears), erythrocytes can

agglutinate in peripheral capillary beds, causing blockage and leading to tissue destruction. Complement-mediated hemolysis can occur as well, provoking intravascular lesions in these regions. Thus, the IgM auto-Abs cause a different type of disease from that caused by IgG warm -reactive auto-Abs.

Drug-induced immune hemolytic anemia occurs when certain drugs associate themselves with erythrocytes. If Abs are formed to the drug's antigenic determinants, the association with red blood cells results in Ab binding to erythrocytes, causing hemagglutination or hemolysis. This leads to anemia. The association with the red blood cell membrane can be either direct binding or indirect adsorption . Direct binding of the antigenic drug to the erythrocytes can lead to Ab binding and complement-mediated cytolysis. Hemolysis can be either due to Ab, specific for the drug, or auto-Ab, specific for certain blood group Ags on the erythrocyte surface. In the latter case, it is believed that direct binding of the drug somehow induces a breakdown in tolerance, leading to the formation of auto-Abs to erythrocyte surface determinants, with subsequent immune lysis of the affected erythrocytes.

In indirect binding, previously formed immune complexes (drug:IgG Ab) can be passively adsorbed onto the erythrocyte, which is an innocent bystander damaged in the subsequent complement-mediated lysis. Many antibiotics, antihistamines, and even aspirin can lead to these immune events. Fortunately, the symptoms usually resolve themselves spontaneously once the person discontinues the use of the drug.

Autoimmune Encephalomyelitis

Encephalomyelitis is a rare disorder of the nervous system that is becoming more common because of the practice of vaccination. Encephalomyelitis refers to an inflammatory condition of the brain and spinal cord. One example is a reaction to a certain type of rabies vaccine. Formerly, if a person was bitten by a rabid animal, vaccine would be given through the so-called Pasteur treatment. The rabies virus was injected into a rabbit, where it multiplied in the brain and spinal cord. The virus subsequently harvested from the rabbit's brain tissue, inactivated, and injected abdominally through a series of injections over a three week period. In some instances, an immune reaction against myelin in neural tissues resulted, thereby inducing autoimmune encephalomyelitis in the treated individual . Rabies vaccines currently in use are safer and do not induce this autoimmune reaction.

Although this treatment immunizes some against rabies, it can produce fatal encephalomyelitis in others. The vaccine is often contaminated with rabbit brain Ags, which can be seen as antigenic by some individuals. Unfortunately, the Abs raised can also cross-react with human brain self-Ags. These crossreacting Abs bind to human brain and spinal cord tissues, fixing complement, destroying the antigenic cells and causing a massive inflammatory reaction with macrophage and lymphocyte infiltration. Brain lesions develop, along with demyelination and axon damage. Death can rapidly ensue from a vaccine-induced autoimmune response.

Autoimmune Thyroid Disease

The thyroid is an endocrine organ that synthesizes hormones, such as thyroxin, that are essential for proper body growth and metabolism. Thyroid diseases fall into three clinical categories: (1) Graves' disease or hypothyroidism(hyperactive) ; (2) Hashimoto's disease, or hypothyroidism (underactive); and (3) myxedema, where there is a near loss of thyroid function. The primary cause for any autoimmune thyroiditis is unknown. Interestingly, there can be a steady progression from Graves' disease to myxedema in the same individual.

With **Graves' disease**, we can find the presence of an auto-Ab to thyroid- stimulating hormone (TSH) receptors can be detected by IF. The thyroid cells are normally stimulated when TSH from the pituitary gland binds to cell receptors. When combined with the thyroid TSH receptor, auto-Ab can also stimulate the thyroid cells to secrete hormones. This would produce the hyperactive thyroid condition of Graves'

disease. Treatment may include surgical removal of the thyroid, with subsequent hormone therapy or use of antithyroid drugs.

The progression to **Hashimoto's disease** involves further complications. Here the thyroid gland becomes infiltrated with lymphocytes and phagocytes, causing inflammation and appearance of a goiter (enlargement of the thyroid). Different auto-Abs appear that can either bind to the protein thyroglobulin or to a cytoplasmic constituent located in thyroid epithelial cells. It is believed that complement-mediated Ab lysis plays a vital role in the destruction of thyroid cells coated with thyroglobullin or thyroid microsomes. In addition, cell-mediated immune responses also influence the course of this disease , as evidenced by the infiltration of phagocytic neutrophils, macrophages, and cytotoxic lymphocytes. Both humoral and cell-mediated reactions lead to the diminished function of the thyroid gland. Antithyroglobulin and antithyroid cell microsomal Abs can be detected by IF or agglutination techniques. Hormone replacement therapy is the primary means of treating Hasimoto's disease.

The symptoms of **myxedema** are manifested because of a near loss of thyroid function and thyroxine synthesis. Because thyroxine plays such an important role in the body's metabolism, lack of this hormone seriously upsets the balance of many bodily processes, including reproductive, immune, cardiovascular, and digestive functions. Myxedemena can be diagnosed by IF techniques detecting the presence of thyroglobulin auto-Abs. It is treated by administration of thyroid extract or synthetic thyroid hormones. If treatment is started soon after the symptoms first appear, recovery can be complete.

The more progressive destruction seen in myxedema seems to involve several immune mechanisms including auto-Ab production, B and T cells, and various Ags released from the damaged thyroid tissue. The cytotoxic responses are so dramatic that they lead to almost complete destruction of the glandular tissue. Histological examination reveals a chronic inflammatory response, with tissue destruction mediated by monocytes and macrophages. Eventual fibrosis occurs. Yet, unlike Hashimoto's disease, in myxedema the thyroid does not become a goiter, rather it shrinks.

Diabetes Mellitus

There are different forms of diabetes mellitus, for example, juvenile and maturity-onset diabetes. In all cases, the hormone insulin is prevented from playing its metabolic role as a mediator of glucose transport across cell membranes. This can comprise basic metabolic functions of cells and, if left untreated, can lead to a number of organ pathologies and even to death. What typifies many forms of diabetes is the pathological role of the immune system, where the formation of auto-Abs can lead to the clinical symptoms.

In one form of **maturity-onset diabetes**, the number of free insulin receptors is reduced due to binding of auto-Ab directed against the Auto-Ab binding to insulin receptor sites can diminish cellular responses to insulin since few sites remain free for the hormone to bind. This insulin-resistant form of diabetes can be treated with injections of insulin at concentrations many times higher than normal in order for the insulin to better compete for receptor sites.

In **juvenile diabetes** also called **insulin-dependent diabetes mellitus**(IDDM), auto-Abs to self-Ags located on the islet cells of the pancreas can lead to cytolysis. These IgG auto-Abs can fix complement and mediate the lysis of the insulin -producing Bcells in the islets of Langerhans of the pancreas. Detection of the auto-Abs can be made by RIA or IF techniques. Several auto-Abs have been isolated. These include a cytoplasic islet cell auto-Ab, and Ab to a 64 kD islet cell protein , and an auto-Ab to insulin. Of these, the 64kD Ab might serve as an important predictor of IDDM since it appears before symptoms of diabetes are expressed..

The cause for loss of self-tolerance is unknown, but it is associated with certain MHC loci and may also be induced by certain enterovirus infections. Certain alleles in the HLA-DQ, HLA-DR3 and HLA-DR4 loci on chromosome 6 appear to be associated with increased susceptibility to IDDM. This locus appears

to be altered in individuals with IDDM.

Goodpasture's Syndrome

The kidney is an organ whose proper functions essential for survival. Chronic renal failure can lead to lethal complications if left untreated. One form of chronic renal failure is rapidly progressive glomerulonephritis resulting from inflammation of the functional unit of the kidney, the nephron. **Goodpasture's syndrome** is a good example of this type of disease. It is a rare autoimmune disease that usually affects young men. is etiology is unknown, but IF techniques demonstrate the presence of autoimmune Abs against the glomerular basement membrane. the glomerulus is a part of the nephron unit that is a capillary network where filtration of the plasma takes place. The IgG auto-Ab can fix complement and so cause necrosis of the glomerulus with loss of renal function. Immune complexes (self-Ag: auto-Ag) forma an even layer on the basal lamina and lead to Type III hypresensitivity reactions. Ig deposits can also found along the basal lamina in the alveoli of the lung, leading to hemorrhages in the lung. thus, it appears that there is crossreaction between the basal lamina of the lung and the kidney's glomerular basal lamina. The treatment of this disease consists of hemodialysis, but individuals often die of lung hemorrhage.

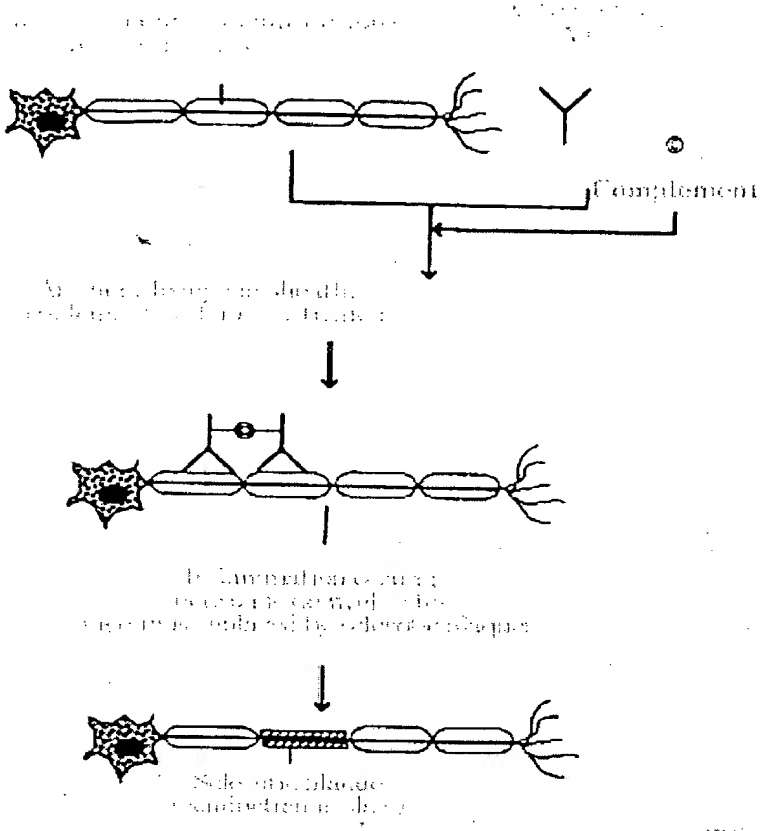
Multiple Sclerosis

Multiple Sclerosis(MS) is a demyelinating disease of the central nervous system (CNS). It is characterized by patchy inflammatory lesions scattered throughout the myelin-containing areas (white matter) of the CNS. Myelin is a fatty, insulating substance that forms an interrupted sheath around certain neurons. It allows for the faster conduction of nerve impulses. In demyelinating disease, the removal of myelin can lead to a slowing down of the nerve impulse in the affected neurons. since temporal and spatial interactions among neurons are common, upsetting the timing in even partially demyelinated neurons can lead to gross neurological dysfunction.

In MS, the demyelination appears to have an immunological, autoimmune component. This is interesting since neural cells do not normally express either class I or class II MHC Ags. The triggering mechanism is not clear, but prior exposure to a virus seems one way autoimmune demyelination might be induced . It has been shown, in some animal systems, that infection with certain viruses insures class I MHC Ags on brain glial cells. Glial cells are accessory cells of the brain that normally do not express these Ags on their surfaces. This induction might occur through soluble factors released by infected glial cells and could set the stage for a breakdown in self-tolerance, leading to virus-induced, immune-mediated demyelination in the CNS.

Demyelination seems to be mediated by immune responses to the self-Ag, myelin. The resulting antimyelin Ab can bind and initiate complement-mediated destruction of the myelin sheath (forming Sclerotic plaques) as seen in early active disease.

AUTOIMMUNE DEMYELINATION IN MULTIPLE SCLEROSIS



Activation of the complement cascade releases fragments C3a and C5a that can mediate an inflammatory response, causing further local tissue damage. Cell-mediated cytotoxic responses also seem to be involved in the immune pathogenesis of this disease in later, older lesions.

There also seems to be an MHC component to this disease. Increased risk is associated with a specific haplotype in the HLA-DR2 region (MHC Class II) located on chromosome 6. If environmental factors, such a as a viral infection, are added to the picture, the risk is statistically greater. It has been suggested that a childhood viral infection may become latent and reappear in adult life to activate MHC-restricted Ags and initiate subsequent responses.

Myasthenia Gravis

The name **myasthenia gravis** means grave muscle weakness. Skeletal muscles thus affected become rapidly fatigued and have a prolonged recovery time. The cause of this disease is unknown. Initial symptoms involve the eye muscles, causing droopy eyelids , with gradual involvement of other skeletal muscles. Death can result if the respiratory muscles become affected. Skeletal muscle cells are innervated by somatic motor neurons that release the neurotransmitter, ACh, into the neuromuscular junction where it diffuses over to the muscle membrane. Located on the skeletal muscle membrane are ACh receptors. When enough of these receptors are loaded with neurotransmitter, a signal can be

initiated, leading to the contraction of the skeletal muscle cell. Rapid destruction of ACh is brought about by a membrane-bound enzyme, cholinesterase, located next to ACh receptor sites. Thus, enough ACh must be released at one time to counteract cholinesterase and load enough receptors to effect a contraction response. In myasthenia gravis, the proper sequence of events are the neuromuscular junction does not occur, leading to the physiological symptoms of aberrant muscle contraction. The basic abnormality is a reduction in the number of ACh receptors on muscle membranes at the neuromuscular junction, brought about by an Ab-mediated autoimmune attack. The auto-Ab formed is against the ACh receptor. These anti-ACh receptor Abs can activate complement, form immune complexes, promote Ab-dependent cellular cytotoxicity(ADCC) responses, and cause endocytosis of these receptors . All of these events lead to the reduction in the number of ACh receptors. If enough receptors are destroyed, muscle contraction cannot be triggered unless cholinesterase activity is inhibited so that receptor sites remaining can be continually stimulated by ACh.

SELECTED AUTOIMMUNE DISEASES: SYSTEMIC DISEASES

Systemic autoimmune diseases can invade many regions of the body. Often circulating immune complexes are deposited in several different organ regions. This occurs when immune complexes become lodged in small capillary beds as the blood flows through the vascular system. Auto-Abs to soluble self-Ags complex with each other and migrate to preferred sites that differ depending on the disease state. Two autoimmune disorders are explored in this section, namely RA and SLE. Immune complexes are preferentially found in the joints of the skeletal system in RA and mainly in the kidney, joints, and skin in SLE. As organ -specific diseases overlap with each other, systemic diseases can too. In fact, RA is frequently associated with SLE. Thus, complexes being deposited systematically to give rise to the more disseminated feature of these nonorganspecific diseases.

Rheumatoid Arthritis

RA is a progressive debilitating inflammatory disease of connective tissues. The most common sites affected by this disease are joints. This disease can be characterized by acute phases, followed by periods of remission. Other organs that can be involved in this systemic disorder include the lung, eye, skin, and nervous system. The course of the disease is variable, but can lead to death in active progressive forms, usually due to infection or complications of therapy.

One diagnostic test for RA is an RIA to detect the presence of RFs (IgG and IgM) in the serum . The cause of the disease is uncertain, but it has been suggested that infection with EBV may lead to activation of a synovial B lymphocytes to produce an abnormal IgG Ab. The immune response to the novel Fc region of this Ig G may be the production of RF, which can subsequently lead to immune-complex formation in the synovial fluid.

RA usually affects the freely movable joints, the ends of the bone are covered with articular cartilage and are held together by a capsule of fibrous tissue called a joint capsule. This joint capsule is composed of an outer layer of ligaments and an inner lining of synovial membrane that secretes synovial fluid, which acts as a joint lubricant. In RA, the formation of immune complexes initiates and amplifies an inflammatory response, causing synovial membrane damage and cell lysis.

Complement fragments, C3a and C5a, have anaphylatoxic and chemotactic properties. The anaphylactic activity leads to the localized release of histamine by mast cells and monocytes, producing symptoms like swelling of joints, redness and pain. Chemotactic factors can cause an influx of phagocytes to the site. These cells can also be provoked to release lysosomal enzymes into the synovial space, which furthers the inflammatory and proliferative response of the synovium.

As inflammation worsens, T and B cells can also be detected and their interaction may ensure the continued production of Igs, continuing the vicious cycle of this immune-complex syndrome. Circulating lymphocytes can enter the joint tissue from venules called the high endothelial venules.

During an acute episode, the proliferating cells of the synovium can grow into the joint cavity and form pannus. Pannus is composed of vascularized fibrous scar tissue that can invade the joint cavity and spread the inflammation to the articular cartilage. The hydrolytic enzymes released can erode the cartilage leading to joint destruction and other complications. There are a number of substances that can activate synoviocytes, including IL-1 and monocyte-derived tumor necrosis factor. Alternatively, the nervous system can also be involved with the release of the neuropeptide substance P, which can stimulate synoviocyte proliferation. This suggest a link between the nervous and immune systems in the expression of this disease state. Substance P is normally involved in the transmission of pain signals, but when released into joint tissue by sensory nerves, can stimulate the release of prostaglandins and collagenase from the rheumatoid synoviocytes. These results can also be obtained from IL-1 and TNF. Perhaps pannus formation is enhanced with the stimulation of synoviocyte proliferation by long term exposure to these immune and neuronals factors.

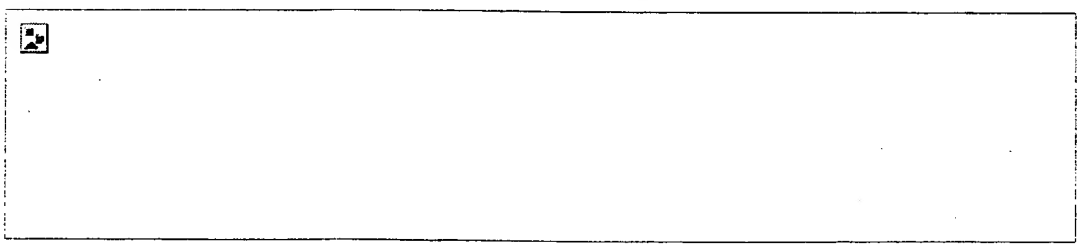
Systemic Lupus Erythematosus

SLE is a multisystem, chronic, autoimmune disease. It involves immunological reactions to a number of self-Ags, resulting in an inflammatory process. The disease mainly affects middle-aged women and is characterized by acute flare-ups and remissions. The prognosis is very individualistic, but death can result if the inflammatory response compromises the function of certain organs, as occurs in lupus nephritis. The name *lupus* (Latin for wolf) was given to describe the characteristic butterfly facial rash, resembling the coloring of a wolf, that can occur. systemic refers to the multiorgan involvement and erythematosus to the redness to the skin rash.

Diagnosis of SLE has traditionally been based on the observation of a characteristic "LE" cell. Immune diagnosis of the disease also can be made by RIA techniques detecting the main auto-Ab in lupus, namely, antinuclear Ab. If kidney involvement is suspected, then IF studies on kidney tissue can reveal "lumpy" deposits of complexes along glomerular basal lamina. Other common symptoms, beside the skin rashes, are pleurisy, arthritic joint pain, and kidney damage.

SLE is a puzzling autoimmune disease because of the great variety of Abs against tissue and cellular components, such as DNA, RNA, and cytoplasmic elements, found in the serum. It is not a monoclonal disease and perhaps reflects a more generalized loss of tolerance to a number of self-Ags, as seen by the wide range of auto-Abs formed. In many individuals, there is a deficiency of Ts cells, which suggests that the loss of tolerance might be due to a release of inhibition imposed by suppressor cells and their products. HLA and viral involvement in triggering the apparent contrasuppression of the B plasma cell system has been proposed. The MHC gene region, HLA-DR3, is suspect in SLE, as are latent viral infections.

The main auto-Ab found in SLE is anti-DNA, which can complex with free DNA to form immune complexes. The resulting circulating immune complexes can become deposited at any number of tissue sites in the body. Once deposited ,the ensuing activation of complement can bring about generalized inflammation ,ADCC and activation of the phagocyte systems. Neutrophils and mononuclear cells can be provoked to release degradative hydrolytic enzymes, which only further the tissue damage until the immune complexes are removed. The disease goes into remission only when synthesis of the auto-Ab downshifts, and the majority of immune complexes are cleared away.



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Vol. 147 No. 2, February 1, 1987

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ARTICLE

HLA immunogenetic heterogeneity in black American patients with Graves' disease

V. Sridama, Y. Hara, R. Fauchet and L. J. DeGroot

Seventy-three American black patients with Graves' disease were typed for HLA-A, HLA-B, and HLA-DR antigens. There was a slight increase in HLA-DRw6 antigen frequency compared with 238 normal American black controls, but this was not significant after correction for the number of antigens tested. A significant increase in HLA-DR4 and HLA-DRw6 frequency was found in a subgroup of patients with exophthalmos (22.9% and 29.2% compared with 7.6% and 10.1% of normal black controls). There was a significant increase in HLA-DRw6 in a subgroup of patients who were thyi antibody-positive (26.0%). The increment in HLA-DRw6 was higher in 32 patients who had both exophthalmos and who were antibody positive (37.5%). A significant increase in HLA-DR5 was f subgroup of patients who did not have exophthalmos and who were antibody-negative (83.3%). findings support previous evidence for immunogenetic heterogeneity in patients with toxic Grave In American blacks HLA-DRw6 is in some way associated with the disease in contrast to the well recognized HLA-DR3 association in whites.

Applicants: Rina Aharoni et al.
U.S. Serial No.: 09/768,872
Filed: June 1, 2004
Exhibit 9

αυτοαντισωμάτων έναντι του υποδοχέα (επαγωγή EAT σε ποντίκια μετά από ανοσοποίηση με συνθετικά πεπτίδια του TSH-R, που αντιστοιχούν σε Τ-επιτόπους).

Με την παρούσα ανασκόπηση καταδείχθηκε η αποφασιστική σημασία των αυτοαντισωμάτων κατά των τριών κύριων αντιγόνων του αδένα στην παθογένεια των αυτοάνοσων παθήσεων του θυρεοειδούς, στη διάγνωση, στην κλινική εικόνα και τη θεραπεία τους, όπως και ο ρόλος αυτοδραστικών Τ-λεμφοκυττάρων έναντι θυρεοειδικών αντιγόνων στην αυτοάνοση διεργασία.

Η σύγχρονη έρευνα στοχεύει σήμερα στην ταυτοποίηση κύριων Τ- και Β-επιτόπων των τριών θυρεοειδικών αυτοαντιγόνων, στη διεξοδική μελέτη νέων θυρεοειδικών αντιγόνων, όπως ο μεταφορέας ιωδίου (NIS, Na^+/I-symporter) και το μεμβρανικό αντιγόνο 64 kD σε συνάρτηση με τη θυρεοειδική οφθαλμοπάθεια, καθώς

και στην ανάπτυξη ενός αξιόπιστου πειραματικού μοντέλου της νόσου Graves. Οι ερευνητικές προσπάθειες επικεντρώνονται παράλληλα στην κατανόηση των παθογενετικών μηχανισμών, με έμφαση στη διερεύνηση της συμμετοχής αποπτωτικών μηχανισμών στην κατασιροφή των θυρεοειδικών κυττάρων από τα Τ-λεμφοκύτταρα, από τα οποία διηθείται ο αδένας στην αυτοάνοση νόσο.

ABSTRACT

Thyroid autoimmunity. Autoantigens, autoantibodies, autoreactive T-lymphocytes, pathogenesis

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Autoimmune thyroid disease has been the paradigm of organ-specific autoimmunity for over 30 years. The high incidence in the general population, the easy access to the target organ and the existence of well established animal models of thyroiditis have facilitated research on the pathogenesis of the disease. Thyroid autoimmune syndromes –Graves' disease, Hashimoto's thyroiditis, primary myxedema or primary hypothyroidism, and postpartum thyroiditis– are characterized by reactivity to self thyroid antigens involving autoreactive T cells and/or circulating autoantibodies. There are three distinct and well characterized thyroid autoantigens: thyroglobulin (TG), thyroid peroxidase (TPO, previously known as "microsomal antigen"), and the thyroid stimulating hormone (TSH) receptor (TSH-R). Autoantibodies to the TSH receptor are responsible for hyperthyroidism in Graves' disease, whereas antibodies to TPO and TG, in high titles, are associated with Hashimoto's disease and primary myxedema. Thyroid autoantibodies are easily measured by simple, sensitive and specific radio- and enzyme-immunoassays. The molecular cloning of genes encoding for all three thyroid autoantigens has had a major impact on the understanding of their autoantigenicity. Knowledge of their primary structures allowed the identification of linear B- and T-cell epitopes through the use of recombinant antigen fragments or synthetic peptides. In general, there is a correlation between these diseases and the genetic loci of HLA-DR and HLA-DQ (HLA class II) regions. Particularly in Caucasians, Graves' disease is related to DR3 haplotype, Hashimoto's thyroiditis to HLA-DR4, DR5 και DQ7, and primary myxedema with HLA-DR5 και DQ7. Research is supported by the development of animal models, spontaneously-developed in certain strains or artificially-induced in normal animals by administration of the relevant peptides. These thyroid-based animal models also constitute precious tools for the study of organ-specific autoimmunity.

Key words: Autoimmune thyroid disease, Autoreactive T-lymphocytes, Thyroid autoantibodies, Thyroid autoantigens

HLA-DRB1 association in Turkish psoriasis vulgaris patients

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Psoriasis vulgaris (PV) is a skin disease present in 1–3% of the Caucasoid population [1]. Although genetic, immunological and environmental factors foster PV, there is evidence that immune system activation plays a central role in keratinocyte hyperproliferation, a typical feature of psoriasis. Moreover, actively dividing T cells accumulate in psoriatic lesions, apart from the presence of a variety of other antigen-presenting, effector and inflammatory cells in psoriatic skin [2]. Peptide presentation by MHC class II molecules is important in human autoimmune diseases. Most of these autoimmune diseases are genetically linked to particular alleles of the class II molecules, and much sequence information has also accumulated on the polymorphisms in these genes. In some autoimmune diseases almost all of the patients carry particular alleles of MHC class II genes (e.g. pemphigus vulgaris). In other autoimmune diseases several different MHC class II alleles carry increased risk, presumably because different MHC class II molecules present the relevant peptide(s) to T cells [3].

Studies have shown psoriasis susceptibity loci on chromosomes as follows: 1, 2, 3, 4, 6th, 8th, 16, 17, 19 and 20. As seen with most autoimmune diseases, psoriasis is found on 6p and associated with allelic component of the major histocompatibility complex [4]. HLA antigens are analysed in different populations and PV has been associated with several HLA class I and class II specifities [5–9].

Among these class I phenotypes Cw6 and B57 have been most consistently reported. The alleles corresponding to these phenotypes are in linkage disequilibrium in normal individuals and compose the proximal class I end of an ancestral haplotype called EH57.1. The class II side of EH57.1 ancestral haplotype contains DRB1* 07 and some other DRB1 alleles [10]. Studies suggest several genetic backgrounds for PV [11]. The first choice is that more than one allele at a single locus may confer susceptibility. The second is that alleles at more than one locus within the HLA region may also confer susceptibility. The true disease locus could be linked to, but separate from, one or more known HLA genes. Although present HLA association information tends to support the last-mentioned hypothesis, it does not in fact rule out the first two. A close linkage between HLA class III and PV has been reported in an Indian population [12].

This study investigates HLA-DRB1 association with PV in a Turkish population.

Material and methods

Patients

The case group of this retrospective association study consisted of 59 unrelated late onset PV patients (33 males and 26 females aged 18–69 years, median age 36.75 ± 12.72) diagnosed and under the surveillance of Istanbul University Faculty of Medicine Dermatology Department. 89 control subjects were also included. The control subjects (47 males and 41 females aged 18–60 years, median age 30.07 ± 12.77) were drawn randomly from volunteer donors of Istanbul University Faculty of Medicine Bone Marrow and Stem Cell Bank.

HLA typing

DNA was extracted from peripheral blood by a standard method [13]. All patients and controls were typed at the Department of Medical Biology, Istanbul Medical School, which is accredited to perform clinical tissue typing by the European Federation of Immunogenetics (EFI). Typing was performed by the sequence specific oligonucleotide primer (PCR-SSOP) method using Dynal RELI SSO HLA-DR. Amplification was performed on a 9700 thermal cycle (PE Biosystems, CA). This is followed by hybridisation; 60 µl denaturation solution was added to each amplified product for 10–15 min at STP to allow for complete denaturation [14]. Dynal AutuReli48 automated machine (Dynal, UK) was used for detection. Hybridisation and citrate buffers are prepared 3h in advance, and substrate and conjugate solutions just before each assay. The PMP5 program was used to interpret the data.

A logistic regression model was used to analyse the data. Significance was taken as $p < 0.05$.

Results

The PV patients in this study had an average age of 36.75 ± 12.72; the minimum age was 12 and the maximum age 69. 55.9% of the PV patients (n: 33) were male and 44.1% (n: 26) female. There were 89 control subjects

Table 1

Logistic regression analysis of HLA-DRB1* alleles in psoriasis vulgaris patients.

HLA-DR	β	Sig.	Exp (β)	Confidence lower	Interval upper
DRB1*01	0.899	0.297	2.456	0.453	13.311
DRB1*15	0.288	0.781	1.334	0.174	10.198
DRB1*16	–0.052	0.961	0.949	0.119	7.578
DRB1*03	1.775	0.013	0.067	1.454	23.937
DRB1*04	–2.135	0.155	0.118	0.006	2.246
DRB1*11	–0.573	0.661	0.564	0.154	2.060
DRB1*13	–1.641	0.030	0.073	0.044	0.850
DRB1*14	–1.426	0.061	0.240	0.054	1.068
DRB1*07	–4.591	0.004	0.010	0.000	0.223
DRB1*08	–1.365	0.069	0.211	0.030	2.164
DRB1*09	2.082	0.926	8.019	0.000	7.58E+19
DRB1*10	–1.021	0.240	0.360	0.066	1.980
DR 52	0.366	0.625	1.442	0.333	6.247
DR 53	2.033	0.130	7.636	0.548	106.419
DR 51	–1.355	0.181	0.258	0.035	1.876
Constant	–3.527	0.445	0.029		

(47 male and 41 female) with an average age of 30.07 ± 12.77 (minimum age was 10 and maximum 60). PV patients had no history of smoking or drug use, nor of viral infection in the last 3 months, so that this group could be used for further research purposes (ie. chromatid exchange studies etc.).

The logistic regression model was used to analyse PV with DRB alleles, and gives a probability estimation of 35–36% for PV occurrence and 84.3% for controls, with an overall probability estimation of 64.9%.

Positive association of HLA-DRB1*03 with PV was observed (Table 1) (n: 12, p: 0.013, 95%, CI: 1.454–23.937) with a relative risk of 2.27 (Table 2). HLA-DRB1*07 association was also observed (n: 14, p: 0.004, 95% CI: 0.000–0.223) with a relative risk of 1.99 (Tables 1 and 2).

Two other HLA-DRB1 alleles have been found to be significantly related with HLA-DRB1 alleles (Table 1). These are DRB1*13 (n: 15, p: 0.030, 95% CI: 0.044–0.850) and DRB1*14 (n: 9, P: 0.061, 95% CI: 0.012–0.498.). The relative risk values for the alleles of HLA-DRB1* 13 and -DRB1* 14 are 1.10 and 1.60 respectively (Table 2).

Discussion

PV is a common HLA-associated skin disease. In the early 1990s suspicions were voiced regarding the presence of a disease gene in the HLA region, on the basis of HLA association studies. The precise genetic basis of HLA association in psoriasis has remained elusive, as it has for other autoimmune diseases [11]. This was due to the fact that little or no evidence was found for linkage to the HLA region. Recent use of genome scans has afforded stronger evidence of linkage to the HLA region [15]. Since HLA associations are well defined for PV and linkage disequlibrium is the most common explanation for allelic association, studies have emerged which reveal susceptibility genes for psoriasis [16]. Candidate psoriasis susceptibility genes have been identified in the HLA region as well as on other genes such as 1p, 2p, 3p, 4q, 8q, 16q, 17q, 19p, 20p. Genome scan studies have provided evidence of linkage to HLA over a rather broad range and not as robust as one might expect, given the strong HLA associations characteristic of PV.

Several HLA phenotypes, corresponding to alleles at the HLA-A, -B, -C, -DRB, -DQA1 and -DQB1 loci have been associated with PV [17]. HLA class II associations have been found in Russian PV patients where DR4 and DR7 antigens were significant [6].

Two susceptible haplotypes were demonstrated as follows in a Thai population [18]. 1) HLA-A2, -B13, -Cw6, -DR7, -DQA1* 0201 and 2) HLA-A2, -B17, -Cw6, -DR7, -DQA1* 0201. HLA-DQB1* 0303 was observed in Japanese psoriatics [19].

PV with early onset and family history is associated with HLA-DRB1* 0701/2, -DQA1* 0201, DQB1 0303. HLA-DRB1*07 provides a risk factor for PV when present with HLA-B27 antigen, and its absence increases the risk of presenting PV while with HLA-DQ3 [20], and HLA risk haplotype Cw 6, DR 7, DQA1* 0201 and HLA-Cw6 has been suggested with reference to the clinical picture of psoriasis vulgaris [21].

The risk we have shown for HLA-DRB1*07 is relatively high (1.99), and statistical significance observed with PV is in agreement with most recent reports.

It is of interest to note that HLA typing using DNA-based techniques gives more precise results [22, 23] than serological methods; the discrepancy was as high as 37% [24]. We have found increased frequency of HLA-DRB1* 14 (n: p <0.005, CI: 0.02–0.498). The association of HLA DRB1*14 is in agreement with a recent report in a Turkish population where only serological methods are used [25]. In this study, in addition to HLA DRB1*14 antigen two other antigen associations with PV were shown for the first time in a Turkish population.

The relation of HLA DRB1*14, -DRB1*13 and -DRB1*03 to progression of PV disease and susceptible haplotypes in Turkish psoriatics has yet to be defined.

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Table 2

HLA-DRB1* allele distribution in controls and psoriasis vulgaris patients.

HLA-DR	Patients n	%	Controls N	%	Probabilty (p)	Relative risk	Confidence lower	Interval upper
DRB1*01	4	6.8	6	6.7	0.993	1.00	0.271	3.730
DRB1*15	15	25.4	19	21.3	0.564	1.44	0.656	3.179
DRB1*16	6	10.2	12	13.5	0.546	0.89	0.307	2.608
DRB1*03	12	20.3	10	11.2	0.127	2.27	0.890	5.789
DRB1*04	8	13.6	23	25.8	0.072	0.47	0.197	1.160
DRB1*11	25	42.4	50	56.2	0.100	0.75	0.388	1.459
DRB1*13	15	25.4	21	23.6	0.800	1.10	0.514	2.369
DRB1*14	9	15.3	9	10.1	0.349	1.60	0.595	4.303
DRB1*07	14	23.7	5	5.6	0.001	1.99	0.850	4.691
DRB1*08	4	6.8	3	3.4	0.345	1.54	0.371	6.437
DRB1*09	0	–	1	0.7	0.312	1.01	0.989	1.034
DRB1*10	4	6.8	4	4.5	0.551	2.08	0.449	9.674
DR 52	42	71.2	68	76.4	0.477	0.73	0.342	1.542
DR 53	22	37.3	35	39.3	0.803	0.90	0.457	1.775
DR 51	21	35.6	26	29.2	0.414	1.318	0.653	2.661

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Common human leukocyte antigen alleles in pemphigus vulgaris and pemphigus foliaceus Italian patients.

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Pemphigus refers to a group of autoimmune blistering skin diseases, mainly identified as pemphigus vulgaris and pemphigus foliaceus, both characterized by the presence of autoantibodies against keratinocyte adhesion molecules, leading to loss of cell-cell adhesion with consequent blister formation. Pemphigus vulgaris is reported to be associated with human leukocyte antigen DR4 and/or DR6 whereas no data are available on pemphigus foliaceus, except for the endemic Brazilian form (fogo selvagem), which is reported to be associated with DR1 and DR4. We here report human leukocyte antigen molecular typing on a total of 87 patients, 61 with pemphigus vulgaris and 26 with pemphigus foliaceus, versus 128 healthy matched controls. Generic typing showed an increase of DRB1*04 and DRB1*14 and a decrease of DRB1*07 in both pemphigus vulgaris and pemphigus foliaceus patients. Molecular subtyping of DR4+ and DR14+ subjects showed a highly significant association between the DRB1*1401 and both pemphigus vulgaris ($p < 0.0001$) and pemphigus foliaceus patients ($p < 0.0001$) together with a significant increase of the linked DQB1*0503 (pemphigus vulgaris $p < 0.0001$; pemphigus foliaceus $p < 0.0001$). Moreover, whereas the association between DRB1*0402 and pemphigus vulgaris ($p < 0.0001$) has been confirmed, no significant association between a specific allele of the DR4 group and pemphigus foliaceus, has been found. Therefore, at least in Italian patients, pemphigus vulgaris and pemphigus foliaceus share DRB1*1401 and DQB1*0503, as susceptible human leukocyte antigen alleles, whereas DRB1*0402 is only found associated with pemphigus vulgaris. The observation that both diseases, pemphigus vulgaris and pemphigus foliaceus, carry the same susceptible human leukocyte antigen alleles has been interpreted as a common genetic background predisposing to pemphigus as, like in other autoimmune disorders, it is not sufficient to explain the onset of the disease on the basis of the sole aforementioned alleles. Other linked genes and/or environmental factors should play a facilitating role in the outbreak of pemphigus, either pemphigus vulgaris or pemphigus foliaceus.

Applicants: Rina Aharoni et al.
U.S. Serial No.: 09/768,872
Filed: June 1, 2004
Exhibit 12

1: Lancet. 1980 May 24;1(8178):1107-9. Related Articles, Linl

Hydralazine-induced systemic lupus erythematosus: influence of HLA-DR and sex on susceptibility.

Batchelor JR, Welsh KI, Tinoco RM, Dollery CT, Hughes GR, Bernstein R, Ryan P, Naish PF, Aber GM, Bing RF, Russell GI.

26 patients with systemic lupus erythematosus (SLE) induced by treatment with the antihypertensive drug hydralazine were investigated to determine if predisposition to the toxic effect was associated with an HLA-DR antigen. 2 of the 26 patients were slow acetylators. The group was compared with three others (1) 113 healthy subjects, untested for acetylator phenotype, (2) 16 slow-acetylator hypertensive patients treated with hydralazine for more than year without developing SLE, and (3) 20 patients with idiopathic SLE. The frequency of HLA-DR4 (73%) was significantly higher in the group with hydralazine-induced SLE than in the other groups (respectively 33%, 25%, and 25%). The ratio of women to men affected was 4:1. If the slow acetylators treated with hydralazine were analysed as one group, it was observed that all women with DR4 developed hydralazine-induced SLE; the only men to do so were those with DR2 who were receiving 200 mg hydralazine per day. These observations have led us to suggest guide lines fo hydralazine therapy and point to a striking association between an HLA-DR antigen and an adverse reaction to a therapeutic agent. It was also noted that the distribution of DR antigens in the hydralazine-SLE patients was significantly different from that in the group with idiopathic SLE. This supports the view that the syndromes are separate entities.

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Exhibit 13

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T suppressor hybridomas and interleukin-2-dependent lines induced by copolymer 1 or by spinal cord homogenate down-regulate experimental allergic encephalomyelitis*

Suppressor T (Ts) hybridomas and interleukin-2-dependent T cell lines were established from spleens of mice, which had been rendered unresponsive to experimental allergic encephalomyelitis (EAE) either by mouse spinal cord homogenate or by the synthetic suppressant copolymer 1 (Cop 1). The Ts hybridoma supernatants and the Ts line cells specifically suppressed the *in vitro* response to the encephalitogenic myelin basic protein (BP), as indicated by inhibition of both the proliferation and interleukin-2-secretion responses of a BP-specific T cell line. Moreover, these Ts cells prevented the development of actively induced EAE *in vivo*. All hybridomas and lines were most effective when injected at the time of disease induction, thus suggesting that they operate as effector suppressor cells, and functionally inhibit encephalitogenic responses. The data presented here suggest that the suppressor cells are stimulated by the protective epitopes included in the BP as well as in the Cop 1 molecules and that they play an active role in the regulation of EAE. The generation of Ts lines and hybridomas, which have been induced by Cop 1, establish the specific stimulation of suppressor cells to EAE as a mechanism underlying the therapeutic activity of Cop 1.

1 Introduction

Experimental allergic encephalomyelitis (EAE), an autoimmune neurological disease, serves as an animal model for human demyelinating diseases, especially multiple sclerosis (MS). The primary immunological mechanism involved are cellular immune responses: encephalitogenic T lymphocytes, specific to myelin basic protein (BP), mediate the pathogenic process [1–3], while suppressor T (Ts) cells play a substantial role in the down-regulation of the disease [4–6]. The use of BP effector clones and lines has provided insight into the cellular mechanisms involved in the pathogenic processes [2, 3]. However, the mechanisms involved in EAE suppression have not been fully elucidated.

Suppressor cells were demonstrated to play an active role in the regulation of several autoimmune diseases [7]. In the EAE system various conditions of unresponsiveness, such as natural resistance in certain strains of mice [4], spontaneous recovery from the disease [5, 6], and induction of tolerance by oral antigen administration [8], have been attributed to the presence of suppressor cells. Suppressor cell activity was also demonstrated in rats and mice rendered unresponsive to EAE by pre-treatment with BP or by the whole spinal cord homogenate [9–11]. Active regulation by suppressor cells has been claimed to operate in human autoimmune diseases as well [7]. The spontaneous remissions and exacerbations often seen in various autoimmune diseases, and particularly in MS, have been explained by changes in the relevant suppressor cell population [12–14]. The accumulated information on suppressor cell involvement in the regulation of EAE and MS, suggests that EAE and other autoimmune diseases may be countered by restoring the balance between the autoagressive and the suppressor cells. Generation and characterization of Ts T cell lines as well as immortalization of suppressor cells by somatic cell hybridization would, therefore, be of paramount importance for further investigations.

Copolymer 1 (Cop 1) is a synthetic basic random copolymer of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine, in a residue molar ratio of 6.0:1.9:4.7:1.0, which exhibits a marked cross-reactivity with the natural autoantigen (BP) and is highly effective in suppressing EAE [15, 16]. The cross-reactivity between Cop 1 and BP was established both on the humoral level [17] and on T cell-mediated immunity [18]. Cop 1 does not exhibit any encephalitogenic activity; however, it exerts a marked suppressive and protective effect on EAE in several species, including primates [11, 15, 16, 19, 20], as well as on chronic relapsing EAE [21]. Cop 1 was also shown to be effective in reducing the number of relapses in early exacerbating remitting MS [22]. Two different mechanisms by which Cop 1 exerts its suppressive activity on EAE have been identified. One involves the inhibition of the effector T cell response that mediates the disease, whereas the other is the stimulation of suppressor T cells that down-regulate the disease. The former mechanism was discovered following the specific *in vitro* inhibition of the response to BP observed in various

[I 10557]

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Abbreviations: **BP:** Basic protein **Cop 1:** Copolymer 1 **MS:** Multiple sclerosis **MSCH:** Mouse spinal cord homogenate

Key words: Suppressor cells / Autoimmunity / Immunomodulation / Experimental autoimmune encephalitis

T cell lines, of either murine or human origin which represent different T cell receptor specificities. It was concluded that Cop 1 inhibits the binding of the encephalitogenic fragment to the MHC [23, 24]. The alternate mechanism underlying the therapeutic activity of Cop 1, namely, the stimulation of suppressor cells, was demonstrated by using the whole spleen cell population from mice rendered unresponsive to EAE by Cop 1. It was shown that T cells are involved in this unresponsiveness which could be adoptively transferred to normal recipients and abrogated by pretreatment with cyclophosphamide [11]. These Ts cells were also shown to regulate the cellular immune response to BP in a specific manner [25]. Moreover, a soluble suppressor factor was extracted from these cells which had the same biological activities as the suppressor cells it originated from, and was capable of interfering with the induction of EAE [26, 27].

In the present study we demonstrated that Ts cell lines and hybridomas could be generated from mice that had been desensitized by either spinal cord homogenate or Cop 1, thus further establishing Cop 1 as a stimulant of suppressor cells specific to EAE, and contributing to the understanding of the mechanism of its therapeutic effect on both EAE and MS. The goal of this study was to generate and characterize murine Ts hybridomas and IL-2-dependent T cell lines, capable of interfering with the various manifestations of EAE *in vitro* as well as *in vivo*.

2 Materials and methods

2.1 Antigens

Mouse spinal cord homogenate (MSCH) was prepared by homogenizing four parts of mouse spinal cord and one part of saline (w/v). The homogenate was strained through a sieve and lyophilized. Basic protein (BP) was isolated from spinal cords of rat (RBP) or mouse (MBP), as previously described [28]. Copolymer 1 (Cop 1) was obtained from either Bio-Yeda (Rehovot, Israel) or Teva (Petach-Tikva, Israel). Purified protein derivative of tuberculin (PPD) was obtained from Statens Serum Institute (Copenhagen, Denmark). Lysozyme from egg-white was obtained from Sigma Chemical Company (St. Louis, MO).

2.2 Mice

The inbred mouse strain SJL/J was purchased from Jackson Laboratories (Bar Harbor, ME). The inbred mouse strains BALB/K and the F_1 hybrid SJL/J × BALB/K were obtained from Harlan-Olac (Bicester, GB). Mice were housed in the department of Laboratory Animals at the Weizmann Institute. Female mice, 7–12 weeks old were used for all experiments.

2.3 EAE

2.3.1 Induction of disease

EAE was induced by injection of 5 mg of lyophilized MSCH, solubilized in PBS, and emulsified in a 1:1 ratio in complete Freund's adjuvant, (CFA, Difco), supplemente with 4 mg/ml of *Mycobacterium tuberculosis* H37R: Difco). The emulsion in total volume of 0.12 ml w: injected into all four footpads. Immediately after, and 48 later, *Bordetella pertussis* toxin, a gift from Pasteur Institut (Paris, France), was injected intravenously.

2.3.2 Assessment of EAE

Mice were examined daily from day 10 post-induction fc signs of EAE and assessed for clinical severity (clinic: score) from 1 to 5 as follows: 1: flaccid tail, 2: hind limb paralyzed, 3: hind and fore limbs paralyzed, 4: tot: paralysis, 5: moribund.

2.3.3 Inhibition of EAE

EAE was induced as described. The preventing treatmen *i.e.* suppressor cells from IL-2-dependent lines or hybrid mas were injected i.v. together, before and after disea induction.

2.4 Suppressor cell induction

Mice were rendered unresponsive to EAE by subcutaneou injection in three to five spots, of either MSCH or Cop emulsified in incomplete Freund's adjuvant (IFA), a previously described [11]. After 15 to 35 days spleen cell were used for establishment of Ts cell lines and Ts hybric omas.

2.5 IL-2-dependent T cell lines

2.5.1 Effector T cell lines

Effector T cell lines were derived from draining lymp nodes and spleens of mice immunized with RBP in enriche CFA, 9–11 days earlier, and selected *in vitro* using eithe RBP or PPD as described by Ben-Nun and Lando [29]. Th cells were propagated with culture medium supplemente with 10 % T cell growth factor (TCGF). Cells were stimu lated by exposure to the desired antigen, presented o syngeneic irradiated (3000 rad) spleen cells (50×10^6/plate for 96 h, every 14–18 days.

2.5.2 Suppressor T cell lines

Spleen cells from mice rendered unresponsive to EAE b Cop 1 in IFA 15–30 days earlier, were cultured with Cop (0.5 mg/plate) following a protocol similar to the on described for effector cells.

2.6 Hybridoma cells

Spleen cells from mice rendered unresponsive to EAE either by MSCH or Cop 1 were used to establish T hybridomas. The cells were first depleted of Ig^+ B cells b absorption on goat anti-mouse Fab antibodies (Bio-Yeda Rehovot, Israel) and then fused in a ratio of 2:1 with th

T cell line BW 5147 (BW) originated from thymic lymphosarcoma of AKR mice, using 41% polyethylene glycol (Serva, 1550). After fusion cells were resuspended in selective HAT medium containing hypoxanthine 100 mM, aminopterine 10 mM, and thymidine 30 mM (Sigma) in culture medium supplemented with 20% FCS. The fused cell mixture was plated in 96-well microculture plates (Falcon), 4×10^4–5×10^4 myeloma cells/well with an additional feeder layer of normal spleen cells (1×10^5/well). The cultures were fed every 5 to 7 days. About 1 month after fusion the HAT components were gradually eliminated from the medium and eventually hybridoma cells were propagated in culture medium with 10% FCS. The hybridoma lines were screened for activity and positive lines were grown on a larger scale.

2.7 *In vitro* assay of T cell reactivity

2.7.1 Proliferation assay of T cell lines and clones

Cells of the T lines or clones were tested for their specific proliferative responses 10–16 days after antigenic stimulation. T cells (1×10^4–2.5×10^4) were cultured with 5×10^5 irradiated (3000 rad) syngeneic spleen cells and various antigen concentrations in a final volume of 0.2 ml in 10% FCS culture medium. At the end of 24–48 h of incubation, cultures were pulsed with 1 μCi of [^{3}H] thymidine (Nuclear Research Center, Negev, Israel). Sixteen hours later, cells were harvested on filter paper and their radioactivity was counted in a beta counter. Results are expressed as mean cpm thymidine incorporation for triplicate cultures.

2.7.2 Assay of IL-2 secretion by T cell lines and clones

The presence of IL-2 in the supernatants of the lines and clones was determined by their ability to support the growth of the IL-2-dependent CTLD cells line [30]. T cells (2.5×10^4) were incubated with antigen and irradiated cells under the same conditions as for the proliferation assay. Twenty-four hours later 50 μl of culture supernatants to be assayed were collected and cultured with CTLD cells (1×10^4/well) at a 1:1 dilution to a final volume of 0.1 ml. After a 24-h incubation period, 1 μCi of [^{3}H] thymidine was added for 5 h and cultures were further processed as described above.

2.7.3 Inhibition assay of T cell response

The T cell lines were inhibited by either hybridoma supernatants, or by irradiated T cells from the IL-2-dependent lines. The inhibitors were added to the assay system together with the stimulating antigen. Inhibition was calculated as:

% inhibition = (1 − cpm in the presence of inhibitor/cpm in the absence of inhibitor) × 100

2.8 Membrane phenotype of T cell lines

Indirect immunofluorescence was performed by incubating 1×10^6 viable cells with 100 μl of rat anti-mouse CD4 mAb (GK3T4 GK15) or anti-mouse CD8 mAb (LyT2 53672) for 30 min. After washing twice in PBS containing 1% BSA and 0.1% sodium azide, FITC-conjugated goat anti-mouse was added for 30 min at 4 °C. The cells were then extensively washed and analyzed by the fluorescence-activated cell sorter (FACSII, Becton-Dickinson, California, USA). In each sample 10^4 cells were counted.

2.9 Data validation

All the results presented in this study have been verified by repeating the experiments at least three times. All the *in vitro* experiments were performed in triplicates. Typical standard deviations were under 10%, and in no case exceeded 20%. A two-sample *t*-test was used to verify the significance of differences between the control and each triplicate average result.

In vivo experiments were performed in groups of 5 to 12 mice. The actual number of animals tested in a specific experiment is stated in each case. The results for repeated, identical experiments are grouped together and the total number of animals stated. A standard normal test for comparing two binomial proportions was used to verify the significance of difference between treated and control mice.

3 Results

3.1 Ts hybridomas

Spleens of mice rendered unresponsive to EAE, either by MSCH or by Cop 1 in IFA, were used as a source enriched in suppressor cells. Six fusion experiments were performed in which suppressor cell-enriched spleen cells were taken from various strains of mice *i.e.*, SJL/J, BALB/K and (SJL/J × BALB/K)F_1 and fused with the BW 5147 (BW), AKR (H-2^k), lymphosarcoma T cell line. More than 2000 hybridoma lines were obtained and screened in an attempt to identify Ts hybridomas.

3.1.1 *In vitro* screening of Ts hybridomas

Preliminary *in vitro* screening for suppressor hybridomas was performed by testing the ability of the hybridoma supernatants to block the specific response of T effector lines to BP, in comparison to their effect on control lines specific to PPD. The results obtained with representative hybridomas are summarized in Table 1. Most of the hybridomas represented here by SJL/J-1-9, BALB/K-90 and (SJL/Jx BALB/K)F_1-1B7, had no significant effect on the response of either T cell line. Others, such as SJL/J-1-20 and BALB/K-42, were nonspecific since they inhibited BP as well as the PPD lines. Fifty hybridomas, represented in Table 1 by SJL/J: 1-17, 3-9, 2-15, BALB/K: 1–10, 2–8, 14, 74, and (SJL/JxBALB/K)F_1: 7-6, 719, 4E7, 4-11, exerted a restricted inhibitory effect on the BP-specific lines while having minor or no effect on the PPD lines. This inhibitory effect was manifested by inhibition of both the antigen-induced proliferation and the IL-2 secretion of the effector T cell lines.

Additional *in vitro* analysis of the Ts hybridomas was performed by testing the direct effect of the specific antigens that had been used for suppression induction (BP and Cop 1) on these hybridomas (Table 2). The proliferation of most of the hybridomas represented here by (SJL/J X BALB/K)F_1-1B7 was not significantly blocked.

Table 1. Inhibition of T cell lines responses by T cell hybrydomas supernatants[a]

Hybridoma no.	Immunizing antigen	[^{3}H] Thymidine incorporation, cpm (inhibition %) BP Line		PPD Line	
		Proliferation	IL-2 secretion	Proliferation	IL-2 secretion
BW		34268 (0%)	ND	14461 (0%)	ND
SJL/J					
1-17	Cop 1	23686 (31%)*	ND	14501 (0%)	ND
3-9	Cop 1	22274 (35%)*	ND	13998 (3%)	ND
2-15	MSCH	18641 (46%)*	ND	13048 (10%)	ND
1-20	Cop 1	18847 (45%)*	ND	8578 (41%)*	ND
1-9	MSCH	27757 (19%)	ND	13357 (8%)	ND
BW		50633 (0%)	5.620 (0%)	101199 (0%)	ND
BALB/K					
1-10	Cop 1	189 (100%)*	41 (100%)*	95228 (6%)	ND
2-8	MSCH	165 (100%)*	57 (100%)*	104359 (0%)	ND
14	MSCH	557 (99%)*	39 (100%)*	102008 (0%)	ND
74	MSCH	18733 (63%)*	3832 (32%)*	102100 (0%)	ND
42	MSCH	141 (100%)*	76 (99%)*	27205 (73%)*	ND
90	MSCH	49265 (3%)	5005 (10%)	101087 (0%)	ND
BW		11504 (0%)	15377 (0%)	7577 (0%)	10002 (0%)
(SJL/J × BALB/K)F_1					
7-6	Cop 1	3879 (67%)*	6437 (58%)*	6993 (8%)	10260 (0%)
719	Cop 1	3273 (72%)*	5490 (64%)*	7133 (6%)	14756 (0%)
4E7	Cop 1	5834 (50%)*	7897 (49%)*	6905 (9%)	12582 (0%)
411	MSCH	5929 (50%)*	9196 (40%)*	6882 (9%)	12976 (0%)
1B7	Cop 1	12642 (0%)	16182 (0%)	7863 (0%)	10923 (0%)

a) T cells (2.5×10^4) were incubated with 5×10^5 irradiated spleen cells and 50 µl of hybridoma culture supernatant. After 24 h, cultures were pulsed with thymidine. The result are presented in cpm and as percent inhibition (in parentheses) of the maximal proliferation response obtained in cultures inhibited with supernatant of the BW control cells. Background proliferation without antigen was 850 cpm for the BP and 537 cpm for the PPD lines.

* Significant inhibition obtained by hybridoma supernatant in comparison to the inhibition obtained by the BW control as indicated by a two-sample *t*-test.

Table 2. Inhibition of the growth of T hybridomas by antigen[a]

Hybrid. no.	Immunizing antigen	[^{3}H] Thymidine incorporation, cpm (inhibition, %)			
			MBP	Cop 1	PPD
BW		116731	123023 (0%)	115851 (1%)	101487 (13%)
BALB/K					
1-10	Cop 1	108842	48246 (56%)*	38703 (64%)*	98706 (9%)
2-8	MSCH	332773	212437 (36%)*	156511 (53%)*	296301 (11%)
14	MSCH	115128	60793 (47%)*	63136 (45%)*	111095 (3%)
74	MSCH	94957	57856 (39%)*	59625 (37%)*	86367 (9%)
42	MSCH	113381	71664 (37%)*	80399 (29%)*	73780 (35%)
(SJL/J × BALB/K)F_1					
7-6	Cop 1	111039	42917 (61%)*	30757 (72%)*	85389 (23%)
719	Cop 1	65045	29106 (55%)*	27133 (58%)*	62486 (4%)
4E7	Cop 1	161056	83335 (48%)*	72814 (55%)*	149896 (7%)
411	MSCH	35663	16983 (52%)*	17933 (50%)*	31085 (13%)
1B7	Cop 1	202113	224792 (0%)	219866 (0%)	220121 (+9%)

a) T hybridoma cells (2.5×10^4) were incubated with irradiated spleen cells and 25 µg antigen/well. After 24 h, direct proliferation was measured by following incorporation of [^{3}H] thymidine. The results are presented in cpm and as percent inhibition (in parenthesis) of hybridoma cell proliferation caused by the antigen in comparison to the proliferation of the same cells without antigen.

* Significant inhibition of hybridoma cell proliferation caused by the antigen in comparison to the proliferation of the same cells without antigen as indicated by a two-sample *t*-test.

Other hybridomas such as BALB/K-42 were inhibited nonspecifically by all the antigens tested including PPD. However, 13 hybridomas were specifically inhibited by BP and Cop 1 but not by PPD, *e.g.* BALB/K: 1-10, 2-8, 14, 74, and (SJL/J X BALB/K)F_1: 7-6, 719, 4E7, 4-11. These hybridomas were blocked by both BP and Cop 1, indicating that they recognized common determinants present on the two antigens. This specific growth arrest was found in all the BALB/K and (SJL/J X BALB/K)F_1 hybridomas that also showed the *in vitro* suppressive activity (Table 1), except for two hybridomas that were only weak inhibitors of BP effector lines. Thus, a high correlation was demonstrated between these two phenomena.

3.1.2 *In vivo* activity of Ts hybridomas

The hybridomas that showed specific suppressive effect *in vitro* were tested for their ability to inhibit EAE *in vivo* (Table 3). Hybridoma cells, 5×10^6, and factors originating from 20×10^6 sonicated cells, were injected together (immediate effect), or a week before (delayed effect) the

Table 3. Inhibition of EAE by T cell hybrydomas[a]

Hybridoma no.	Hybridoma specificity	Immediate		Delayed	
		Incidence of disease	Clinical score	Incidence of disease	Clinical score
SJL/J					
Control		32/42 (76%)	2.0	32/42 (76%)	2.0
BW		26/34 (76%)	2.2	18/24 (75%)	2.0
1-17	Cop 1	3/7 (43%)*	0.9	4/4 (100%)	3.5
3-9	Cop 1	2/5 (40%)*	0.4	4/4 (100%)	3.2
2-15	MSCH	2/5 (40%)*	0.4	2/4 (50%)	1.6
1-9	MSCH	7/7 (100%)	3.4	6/6 (100%)	4.3
BALB/K					
Control		9/12 (75%)	1.8	9/12 (75%)	1.8
BW		6/10 (60%)	1.2	7/10 (70%)	1.4
1-10	Cop 1	2/10 (20%)*	0.4	ND	
2-8	MSCH	2/11 (18%)*	0.4	ND	
14	MSCH	0/11 (0%)*	0.0	3/6 (50%)	1.0
74	MSCH	0/11 (0%)*	0.0	3/4 (75%)	1.2
(SJL/J × BALB/K)F_1					
Control		8/16 (50%)	1.6	ND	
BW		5/14 (36%)	0.9	ND	
7-6	Cop 1	0/11 (0%)*	0.0	ND	
719	Cop 1	0/15 (0%)*	0.0	ND	
4E7	Cop 1	0/13 (0%)*	0.0	ND	
4-11	MSCH	1/16 (6%)*	0.1	ND	
1B7	Cop 1	3/5 (60%)	2.2	ND	

a) Hybridomas were tested in (SJL/J × BALB/K)F_1 mice. Controls and BW-treated controls comprise the combined results of several experiments, as variability was less than 20%.

* Significant inhibition of disease incidence by hybridomas in comparison to mice treated with the BW control cells as indicated by a standard normal test.

Table 4. Specificity of Ts cell line

Antigen in culture	[^{3}H] Thymidine incorporation (cpm)					
	F_1-Ts-30D + APC from			F_1-Ts-Lys + APC from		
	F_1 ($H\text{-}2^{s,k}$)	SJL/J ($H\text{-}2^s$)	BALB/K ($H\text{-}2^k$)	F_1 ($H\text{-}2^{s,k}$)	SJL/J ($H\text{-}2^s$)	BALB/K ($H\text{-}2^k$)
	540	5295	411	798	207	321
Cop 1	114782	96708	113318	1564	262	769
RBP	815	15170	324	415	240	294
MBP	2633	35999	4140	898	244	357
Lysozyme	530	3338	451	82051	1392	10885
Con A	181414	124685	131826	243847	145443	185164

a) T cells (2.5×10^4) were incubated with 5×10^5 irradiated spleen cells and 10 μg/well of antigen. After 24 h cultures were pulsed with [^{3}H] thymidine and harvested 16 h later.

induction of EAE. A few hybridomas of SJL/J origin had some effect on the development of the disease, (SJL/J: 1-17, 3-9 and 2-15). Mice injected with these hybridomas still showed at least 40 % incidence of disease in comparison to 76 % in control mice or those injected with the BW cells, namely, less than 50 % inhibition of disease. Hybridomas of BALB/K origin, a naturally EAE-resistant strain, caused a much more significant inhibition. Two of the four presented in the Table, *i.e.* BALB/K: 14 and 74, completely prevented the disease (no animals sick out of 11 mice injected). From the (SJL/J × BALB/K)F_1 fusion, nine hybridomas, represented here by (SJL/J × BALB/K)F_1: 7-6, 719, 4E7 and 4-11 also exerted significant EAE inhibitory activity. All the hybridomas were effective when injected at the time of disease induction (immediate effect) and not when injected a week before (delayed effect).

3.2 Ts cell lines

An additional approach to obtaining suppressor cells for EAE was to grow IL-2-dependent Ts cell lines. Such a line was developed from spleen cells of (SJL/J X BALB/K)F_1 mice that had been rendered unresponsive to EAE with Cop 1 in IFA 23 days earlier, and was denoted F_1-Ts-30D. A control line was established from mice injected with lysozyme (a control basic antigen), in the same way and denoted F_1-Ts-Lys.

3.2.1 Reactivity of F_1-Ts-30D and F_1-Ts-Lys lines

The *in vitro* reactivity of these two lines was tested in lymphocyte transformation assay, using antigen-presenting cells (APC) from SJL/J, BALB/K and their F_1 hybrids. The results summarized in Table 4 show that F_1-Ts-30D was highly reactive with Cop 1, and similar responses were obtained with the three sources of APC. Although the cells originated from mice injected only with Cop 1, they cross-reacted with BP (RBP and MBP), and this response was restricted to SJL/J APC. The F_1-Ts-Lys line responded exclusively to lysozyme and the response was clearly linked to H-2^k.

The F_1-Ts-30D line showed no anti-idiotypic response when co-cultured with the syngeneic, encephalitogenic, BP-specific line, F_1-SP-RBP, as a target (data not shown).

Using FACS analysis, the F_1-Ts-30D, F_1-Ts-Lys and the F_1-SP-RBP lines, were identified as $CD4^+$ cells, which do not express the CD8 marker.

3.2.2 Suppressive activity of F_1-Ts-30D line *in vitro*

The F_1-Ts-30D line was tested for its ability to inhibit *in vitro* the response of the BP-specific T encephalitogenic line F_1-SP-RBP to BP. As can be seen in Fig. 1 the F_1-Ts-30D line significantly inhibited the response of the F_1-SP-RBP lines to RBP, as measured by both direct proliferation, up to 66 % inhibition – (Fig. 1A) and IL-2 secretion, 50 % inhibition (Fig. 1C), while the F_1-Ts-Lys line when similarly tested had no inhibitory effect (Fig. 1B and 1D, respectively). The inhibition was proportional to the number of the inhibiting F_1-Ts-30D cells, and the highest inhibition was obtained in a 1:1 ratio of suppressor to effector cells. These results indicated that F_1-Ts-30D line suppressed the response to the autoantigen BP *in vitro*.

3.2.3 Suppressive activity of F_1-Ts-30D line *in vivo*

The development of EAE was almost completely inhibited by 10^7 activated F_1-Ts-30D cells (Fig. 2). Only 1 animal from a group of 11 mice was sick, in comparison to 60 % morbidity in the control mice injected only with MSCH

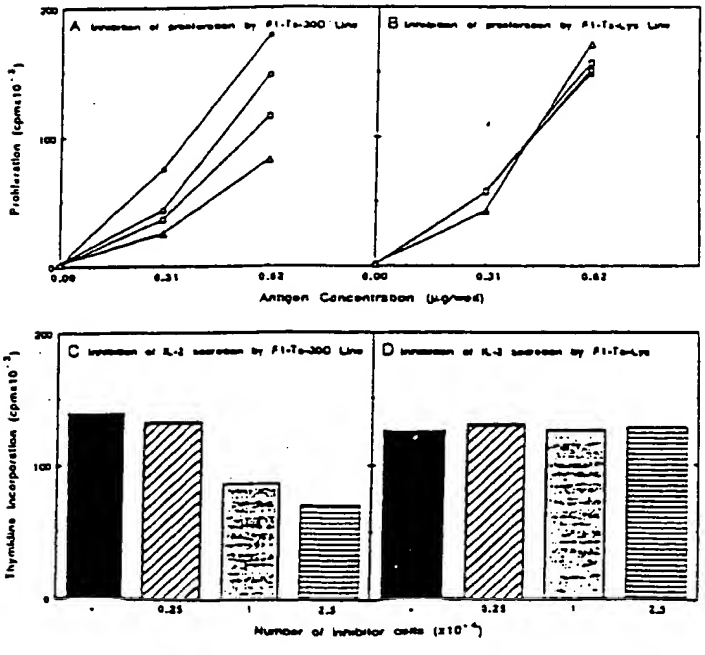

Figure 1. Suppressive activity of the F_1-Ts-30D line on the response of the F_1-SP-RBP encephalitogenic line to RBP. Encephalitogenic F_1-SP-RBP cells (2.5×10^4) were incubated with activated F_1-Ts-30D (A, C) or F_1-Ts-Lys (B, D) cells, irradiated (1000 rad) and with RBP. Inhibition of F_1-SP-RBP line was measured by proliferation assay (A, B) and IL-2 secretion (C, D). The inhibiting cells were cultured in concentration of: no inhibiting cells (●), 2.5×10^3 (○), 1×10^4 (□) and 2.5×10^4 (△). Antigen concentration used for induction of IL-2 secretion (C, D) was 0.62 µg/well).

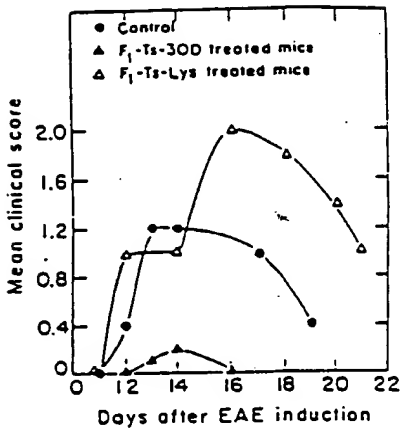

Figure 2. Suppressive activity of F_1Ts-30D line in *in vivo* inhibition of EAE. Activated F_1-Ts-30D cells (10^7, ▲), or F_1-Ts-Lys cells (10^7, △), were injected into (SJL/J × BALB/K)F_1 mice on the day of disease induction. Control mice were injected only with MSCH for induction of disease (●). EAE was induced as described in Table 3.

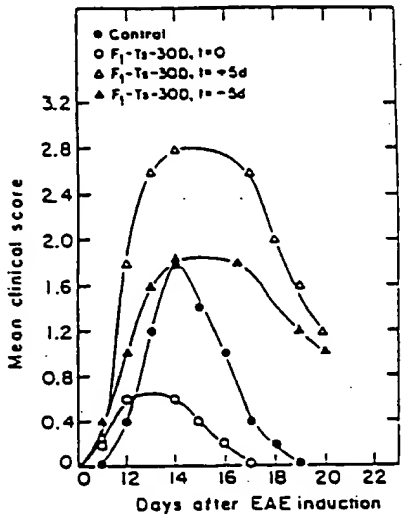

Figure 3. Kinetics of F_1-Ts-30D suppressive activity 6×10^6 activated F_1-Ts-30D were injected into (SJL/J × BALB/K)F_1 mice at days: −5 (▲), 0 (○), or +5 (△), to EAE induction in comparison to control mice (●).

disease-inducing inoculum (85 % inhibition). The protection caused by F_1-Ts-30D cells was also reflected in decreased severity (83 % suppression), as well as in a delay in the onset of the disease (1 day). The suppression obtained was proportional to the number of cells injected, as 5×10^6 F_1-Ts-30D cells had only a slight protective effect (not shown). No inhibition of EAE was observed in mice injected with 10^7 activated F_1-Ts-Lys cells. This line even caused nonspecific stimulation of the disease, indicating that the suppression caused by the Cop 1 line F_1-Ts-30D was specific.

3.2.4 Kinetics of F_1-Ts-30D suppressive activity

To study the kinetics of the suppression caused by the F_1-Ts-30D line, 6×10^6 activated cells were injected either 5 days before (−5), 5 days after (+5), or on the same day of disease induction (0). As illustrated in Fig. 3, injection of the suppressor cells at day 0, *i.e.* on the day of EAE induction, seemed to be optimal, as cells injected on either at days −5 or +5 were incapable of inhibiting EAE, thus suggesting that these suppressor cells are effector suppressor' cells.

Two additional Ts cell lines were established in a similar procedure to the F_1-Ts-30D line and were found to have similar suppressive effects on EAE manifestations both *in vitro* and *in vivo*. These results emphasize that Cop 1 does indeed induce suppressor cells which mediate protection against EAE.

4 Discussion

The goal of the present study was to obtain a homogenous population of suppressor cells that will interfere with the various manifestations of EAE and restore the self-tolerance equilibrium that is hampered in the pathological process. To this end, both Ts cell hybridomas and IL-2-dependent cell lines were established from spleen cells of mice rendered unresponsive to EAE by the injection of either spinal cord homogenate or the EAE suppressant copolymer, Cop 1.

The hybridomas obtained exhibited a specific functional *in vitro* suppressive effect on the response to BP, as was measured by testing their supernatant activity on either the direct proliferation or the IL-2 secretion of effector T cell lines (Table 1). Interestingly, we have noticed that the hybridomas which blocked the specific response to BP were by themselves blocked by either BP or Cop 1, but not by PPD (Table 2), and a high correlation was demonstrated between these two phenomena. Blocking of hybridoma growth, as monitored by [^{3}H]thymidine incorporation following antigen-specific activation, has been reported by others [31, 32].

The most meaningful test for suppressor cells in the present context is the ability to suppress the disease *in vivo*, and indeed a significant suppressive effect on EAE could be demonstrated by several of the hybridomas generated in this study. The most potent suppressor hybridomas were those originating from either BALB/K or (SJL/J × BALB/K)F_1 mice, whereas those obtained from SJL/J mice were less effective. The BALB/K resistant strain had been shown to maintain a natural high level of suppressor cells which prevent the manifestation of disease [5], while the SJL/J mice that are susceptible to EAE have some malfunction of the suppressor cell arm [33, 34]. This may account for the differences observed between the activity of the suppressor hybridomas originiating from these strains.

The suppressive activity demonstrated by the hybridomas both *in vitro* and *in vivo* indicates that these hybridomas are indeed suppressor hybridomas that can down-regulate EAE. To our knowledge, this is the first report on suppressor hybridomas in the EAE system. Such hybridomas constitute a very useful tool for research and an easy

source to obtain and purify large quantities of cellular factors. However, the disadvantage of using malignant lines when considering a therapeutic method, have led us to use a more natural approach for obtaining suppressor cells for EAE, namely the establishment of an IL-2-dependent suppressor line.

IL-2-dependent suppressor lines, *e.g.* the F_1-Ts-30D, were generated from spleen cells of mice that had been induced to be unresponsive to EAE by prior injection of Cop 1. This Cop 1-specific line suppressed in a dose-dependent manner the *in vitro* response of an encephalitogenic line to RBP, as demonstrated by inhibition of both the proliferation and the IL-2 secretion of the effector cells, while no suppressive effect was observed when co-cultured under the same conditions with a lysozyme-specific control line (F_1-Ts-Lys) (Fig. 1). Furthermore, the F_1-Ts-30D cells were very effective *in vivo* as well, and prevented almost completely the development of EAE, as reflected in both the incidence and the clinical score of the disease (Fig. 2). As demonstrated for the *in vitro* reactivity of the F_1-Ts-30D line, the *in vivo* effect was also dose dependent and specific, since no inhibition was observed in mice treated with the lysozyme control line. The lysozyme line even caused a nonspecific stimulation of the disease. This enhancement of EAE by co-injection with activated non-relevant cells or by relevant cells before or after EAE induction, could be attributed to nonspecific penetration of the CNS [35] and possible induction of IFN-γ or other cytokines that might have stimulated MHC expression [36]. Since F_1-Ts-30D-injected cells had also been activated, the appropriate control for their functional activity should be treatment with activated F_1-Ts-Lys cells. Such a comparison emphasized even more the suppressive effect demonstrated by the Cop 1-specific line.

The F_1-Ts-30D line expressed the CD4 marker. Although traditionally suppressor cells were considered to express the CD8 phenotype, CD4 suppressor T cells are not without precedent [37] and $CD4^+$ Ts cells and lines were also demonstrated in the EAE system [38, 39].

The EAE suppression exerted by both the hybridomas and the IL-2-dependent line was most effective when the Ts cells were injected at the time of disease induction indicating that these are effector suppressor cells that directly suppress the pathogenic cells without involving induction of an additional subset of suppressor cells, such as anti-idiotypic cells [40] and that suppressor T cells play an active role in the regulation of EAE.

Cop 1 was as efficient as BP in inducing suppressor T hybridomas, as reflected in both the number of "positive" suppressor hybridomas, and the extent of the suppression induced by these hybridomas, *in vitro* as well as *in vivo*. It can, therefore, be concluded that Cop 1 contains region(s) that induce suppressor cells to EAE, i.e. suppressive determinant(s). Moreover, these suppressive determinants are shared by BP and Cop 1 as indicated by the following experimental results: The hybridomas and the IL-2-dependent T cell lines that had been induced by Cop 1, inhibited the stimulation of BP-specific effector lines by BP (Table 1, Fig. 1). A similar degree of hybridoma growth arrest was induced by BP and Cop 1 (Table 2). In addition, F_1-Ts-30D – the Cop 1-specific line cross-reacted with BP when presented on SJL/J macrophages (Table 4). Finally, suppressor cells specific to Cop 1 prevented the *in vivo* induction of EAE, where the main encephalitogenic agent is BP [1–3]. Hence, the cross-reactivity between BP and Cop 1, previously demonstrated on both the humoral and the cellular levels, was found here for a specific subset of T cells – the suppressor cells.

These suppressor cells probably recognize an epitope present on Cop 1 and BP which is different from the encephalitogenic determinant. This "suppressive or protective epitope" selectively stimulates Ts rather than T helper lymphocytes. The concept that T helper and Ts repertoires differ, is based on the observation that suppressor T cells specific for one antigenic determinant can inhibit the T and B cell response to other determinants on the same molecule [41–43]. Such suppressive determinants have been demonstrated for BP and shown to protect against EAE in guinea pigs [44] and rats [40, 45]. The availability of Ts hybridomas and lines such as those described herein may provide a useful tool for understanding the immune regulation of EAE. The use of Cop 1, which contains only the suppressive determinants of BP for induction of suppressor cells, is of unique advantage.

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